Chemotaxis and inhibition of the kauri killer, *Phytophthora agathidicida*

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A thesis presented for the degree of

Master of Science

At the University of Otago,

Dunedin, New Zealand

June 2018

Abstract

Phytophthora are devastating pathogens which cause diseases in thousands of economically and ecologically important plants worldwide. Often referred to as 'fungus-like', *Phytophthora* are a genus of eukaryotic oomycetes. There are over 100 species of *Phytophthora* and all are pathogenic to plants. *Phytophthora agathidicida* is one species which is of great concern here in New Zealand, as it infects native *Agathis australis* (kauri) causing kauri dieback disease. Kauri forests have been greatly reduced over the past 200 years, resulting in low and fragmented genetic diversity of kauri. The remaining kauri are now under serious threat from *P. agathidicida* and could be extinct if intervention is not taken.

Phytophthora have several life cycle stages, some of which are not found in most true fungi. In addition to a mycelial growth phase (similar to fungi), *Phytophthora* produce zoospores, which are key to the epidemic spread of disease, facilitating host-to-host transmission. In this thesis, a variety of approaches have been used to explore the different lifecycle stages of *P. agathidicida*. An improved method for zoospore production was developed, which facilitated further study of this emerging pathogen.

The chemotactic behaviour of *P. agathidicida* zoospores was studied for the first time in the research described in this thesis. Initial chemotaxis assays showed *P. agathidicida* zoospores were attracted to a variety of amino acids and sugars. *P. agathidicida* also exhibited increased chemotaxis to amino acids at pH 3.0, a pH level similar to the soil conditions around mature kauri.

Currently there is no cure for kauri dieback; therefore a chemical treatment for *P. agathidicida* is vital for the survival of kauri. In the research conducted in this thesis a high throughput screen was developed for anti-oomycete compounds as a potential new tool for finding a cure to kauri dieback. Over a hundred compounds were efficiently screened for mycelium inhibition and any resulting compounds were then tested on multiple *P. agathidicida* life cycle stages, including zoospore motility and germination. Copper salts and a quaternary ammonium salt were the most effective across all three life stages, indicating their potential application to help manage the disease. Overall, the improved methodologies, and fundamental insights from this work will help to inform further efforts to control the disease.

Acknowledgements

First and foremost, I would like to thank my supervisor, Monica Gerth, without your guidance this thesis would not have been possible. Your enthusiasm and kind support was particularly key for getting me through this period. You always encouraged the best out of me, and for that I am very grateful.

I also want to say a big thank you to Scott Lawrence; this thesis would also not have been possible without your help. Thank you for the great chats and constant support throughout this often weird but wonderful research.

I want to extend a thank you to the E^3 family and a special thank you to Wayne Patrick and Monica for creating such wonderful working environment; you made my post graduate adventure at Otago a great experience. Thank you for the support, the laughs, the parties and the lab tramps. You have all set my bar high for future lab groups.

To my parents and brother, thank you for the never-ending support and constant belief in me. Your reassuring phone calls and your abilities to always make me laugh would lift up my spirits every time. I would not be where I am without you and for that I am forever thankful.

A mountain of gratitude must also go towards all those who have shared a rope, climbed a mountain, crossed a river and tramped a track with me over the course of my Masters. Those trips and climbs replenished my soul when times were tough. Finally, a special thank you of course goes to Chris, who gave unconditional encouragement throughout this period; you endured the lows and celebrated the highs with me, thank you.

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A List of Abbreviations

AIM	Auto Induction Media
cV8	Clarified V8 juice
EC_{50}	Concentration of a drug that gives half-maximal response
GPCR	G-protein coupled receptors
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	KiloDaltons
LB	Luria-Bertani
OD_{600}	Optical density at 600 nm
PARP	*Phytophthora*-selective agar
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PIPK	Phosphatidylinositol phosphate kinase
PM Plates	Phenotype Microarray plates
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOC	Super optimal broth with catabolite repression
TAE	Tris-Acetate EDTA
TB	Terrific Broth
v/v	Volume/volume
w/v	Weight/volume

Chapter 1

Introduction

1.1 *Phytophthora*

1.1.1 The Global Plant Destroyers

Phytophthora are a genus of eukaryotic oomycetes, commonly referred to as water moulds (Judelson & Blanco, 2005). They resemble filamentous fungi but are actually more closely related to diatoms and brown algae (Kamoun *et al.*, 2014). The name of the *Phytophthora* translates to "the plant-destroyers" in Greek and are known to destroy thousands of plants across the globe, threatening economically, ecologically and culturally important plant and crop species (Erwin & Ribeiro, 1996).

There are over 100 species of *Phytophthora,* all of which are pathogenic to plants (Kroon *et al.*, 2012). Some species of *Phytophthora* have a broad host range and will infect a wide variety of plants; *Phytophthora cinnamomi* for example can infect approximately 5000 plant species and therefore earns its name as the biological bulldozer (Zentmyer, 1980). Others are host-specific and will only infect their host or similar host plants.

One of the most infamous *Phytophthora* species is *Phytophthora infestans*, the causative organism of the late blight potato disease. This disease eradicated potatoes, a vital food crop in Ireland in the 1840's. This resulted in mass starvation and emigration causing economic and social chaos (de Bary, 1876). Even today the economic damage of crops caused by *Phytophthora* in the United States alone is in the billions of dollars and worldwide the estimated effect is many times this amount (Erwin & Ribeiro, 1996). *Phytophthora sojae* for example is host-specific to soybean crops, destroying the roots of the crop and causing an estimated loss worldwide of 1-2 billion US dollars per year (Tyler, 2007). As well as their agricultural impact, *Phytophthora* have a devastating impact on natural ecosystems. For example, *P. cinnamomi* is currently destroying vast amounts of native flora in Western Australia (Hardham & Blackman, 2018).

1.1.2 The Impact of *Phytophthora* in New Zealand

Phytophthora are also having a deleterious impact in New Zealand. A large proportion of New Zealand's tradable exports are agricultural and forestry products which have a worth

in the billions of dollars per annum. Therefore protecting these assets from plant pathogens such as *Phytophthora* is of high economic importance. *Phytophthora pluvialis* for example is infecting *Pinus radiata* (Dick *et al.*, 2014), the backbone tree of New Zealand's forestry and *P. cinnamomi* is infecting avocado crops, reducing yields by up to 20% per annum (West, P., personal communication, New Zealand Avocado Growers' Association). The origin of most *Phytophthora* species in New Zealand is unknown; however most are likely to have originated outside of New Zealand (Newhook, 1972).

The focus of this Masters research is on a recently emerged *Phytophthora* in New Zealand, *Phytophthora agathidicida,* which causes kauri dieback disease (Weir *et al.*, 2015). This disease is threatening the existence of native *Agathis australis* (kauri). Currently, there is no cure for kauri dieback, therefore research into *P. agathidicida* is vital for the survival of kauri.

1.2 The Life Cycle of *Phytophthora*

Phytophthora are well adapted to diverse environments and plants (Erwin & Ribeiro, 1996). The life cycle allows the organisms to produce different asexual and sexual spores that are specialised for survival, dispersal and infection (Judelson & Blanco, 2005).

In the vegetative state, the thallus of Phytophthora is called mycelium which consists of a net of intertwined tubular structures called hyphae. In an infected plant the hyphae will directly invade the plant cells to obtain nutrients, thus causing necrosis of the plant tissues (Erwin & Ribeiro, 1996). Most Phytophthora will infect at the roots, causing root rot and thus cutting off nutrient supplies to the rest of the plant which leads to canopy wilting or death if the damage is done on the major roots. Infection that progresses to the main stem causes visible cankers to form on the bark of trees. Some species of Phytophthora cause multiple symptoms in a single host and others have delayed symptoms but most species cause symptoms similar to abiotic factors, injury or other plant pathogens, therefore diagnosis often requires expert analysis (Tsao, 1990).

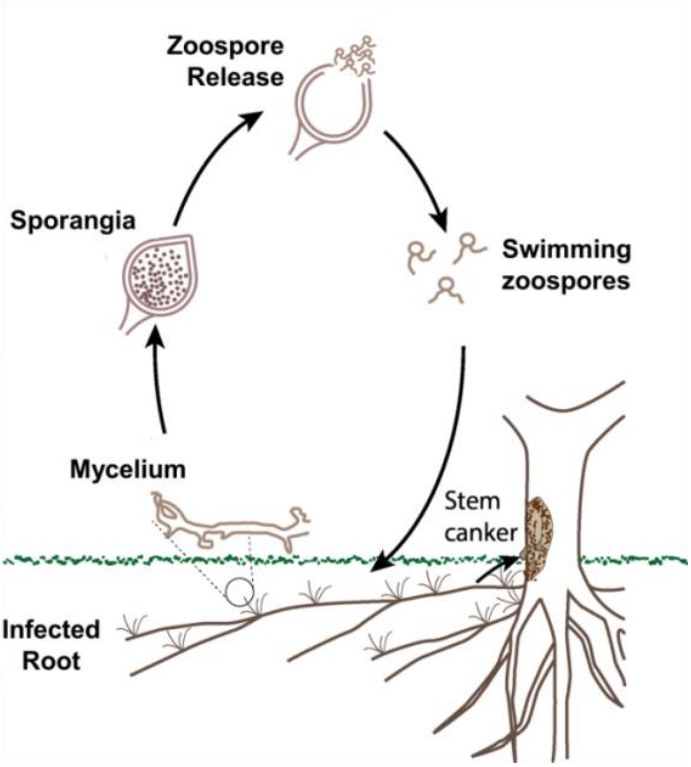

Figure 1.1: Summary of *Phytophthora's* asexual infectious life cycle.

As an infected plant declines in health, spores are required to move the pathogen to a new host (Figure 1.1). *Phytophthora* produce several types of spores: sporangia, zoospores, chlamydospores and oospores (Judelson & Blanco). Sporangia are generated asexually and bud off from the termini of hyphae (Judelson & Blanco, 2005). Sporangia vary in shape and size but often are ellipsoid or pear shaped with a papilla at the tip (Erwin & Ribeiro, 1996). Sporangia are produced under suitable environmental conditions. These conditions vary between the species and recreating the correct conditions *in vitro* can be difficult for some species of *Phytophthora* (Erwin & Ribeiro, 1996). Sporangia are important for the virulence of the pathogen because they contain zoospores, which are needed to infect the next host (Figure 1.1).

Zoospores are unicellular kidney shaped spores with two heterokont (uneven) flagella which allow the cells to be motile (Erwin & Ribeiro, 1996). Zoospores are released from the sporangium into the environment where they go on to infect the next host (Figure 1.1). They are short lived but can swim for several hours or even days (Judelson & Blanco,

2005). In nature they can swim through water logged soils at a rate of up to 7 mm a minute (Young *et al.*, 1979). Once the zoospores stop swimming they encyst (develop a cell wall) and these encysted zoospores geminate and form hyphae which allows the *Phytophthora* to infect and grow within a plant host and thus complete its life cycle (Figure 1.1). Zoospores are required to remain metabolically active to be able to sense and respond rapidly to surrounding chemical signals, however this also depletes nutrient reserves making zoospores vulnerable to the environment (Judelson & Blanco, 2005).

Phytophthora also produce other spores called chlamydospores and oospores during its life cycle. Oospores are produced by sexual reproduction; they are spherical and thick walled which enables them to survive long term in soil or plant tissue. Chlamydospores are another spherical spore with slightly less thick walls and are produced asexually. Both oospores and chlamydospores can germinate to form sporangia and are also known to be long-term survival structures which can survive through harsh environmental conditions (Erwin & Ribeiro, 1996).

The physiology of spore production is complex and still not fully understood (Erwin & Ribeiro, 1996). Research into spore pathways will increase the understanding of the *Phytophthora* and could also lead to effective strategies for controlling *Phytophthora* diseases. Sporangia and zoospores for example are particularly vulnerable to chemical treatments as they can only briefly survive outside a host and have restricted nutrient reserves that limit their ability to resist toxins (Judelson & Blanco, 2005).

1.3 *Phytophthora* and Fungi

Phytophthora were originally classified as fungi due to their similarities in filamentous growth and sporulation (Latijnhouwers *et al*, 2003). Due to their similarities some of the more broad ranged fungicides can be used to control *Phytophthora* (Akrofi *et al.* 2013; Elliott *et al.* 2015; Saville *et al.* 2015).

Despite the morphological similarities between *Phytophthora* and fungi, they have substantial differences. *Phytophthora* for example produce free swimming spores called

zoospores and also have cell walls composed of cellulose, not chitin (Table 1.1; Judelson & Blanco, 2005). *Phytophthora* are not phylogenetically close to fungi but are in fact closer to brown algae and diatoms (Table 1.1; Kamoun *et al.*, 2014). *Phytophthora* are also diploid whereas fungi are normally haploid (Table 1.1; Judelson & Blanco, 2005), thus making *Phytophthora* more versatile and adaptable which has enabled *Phytophthora* to develop resistance to antibiotics and other common fungicides such as metalaxyl (Erwin & Ribeiro, 1996). Due to these differences, not all fungicides can target *Phytophthora*. For example *Phytophthora* are insensitive to polyoxin fungicides, which inhibits the synthesis of chitin for cell walls. *Phytophthora* are also insensitive to fungicides that inhibit sterol synthesis because *Phytophthora* do not require sterols for growth (Erwin & Ribeiro, 1996).

Table 1.1: Major differences between oomycetes and fungi (Judelson & Blanco, 2005).

Feature	Oomycete	True fungi
Neighbouring taxonomic groups	Diatoms and brown algae	Animals
Hyphal architecture	Aseptate and coenocytic tubular hyphae	Either single cell or septated hyphae, with one or more nuclei per compartment
Ploidy of vegetative hyphae	Diploid, except for transient haploid nuclei in gametangia	Typically haploid or dikaryotic; often with a stable or semi-stable diploid stage following mating
Typical size of genome	50–250 Mb	10–40 Mb
Major glucans in cell walls	Cellulose (β-1,4-linked glucose), and β-1,3, and β-1,6-linked glucose polymers	Usually chitin (β-1,4-linked N-acetylglucosamine) and/or chitosan (β-1,4-linked glucosamine), often with other β-1,3, and β-1,6 glucans
Pigmentation	Usually unpigmented	Very common in hyphae or spores, or secreted (melanin, carotenoids)
Toxic secondary metabolites	None described	Common (typically aromatic, heterocyclic compounds)
Mating hormones	Non-peptide, probably lipid-like	Usually small peptides or lipopeptides
Predominant asexual spore	Undesiccated, unicellular sporangia (multinucleate cells)	Desiccated single or multicellular conidia (one nucleus per cell)
Motile asexual spores	Nearly universal, biflagellated zoospores	Uncommon, only in chytrids, which are monoflagellate
Sexual spores	Oospores, formed on the termini of specialized hyphae each containing one viable zygotic nucleus	Various types, often formed in large numbers within complex enclosures (such as, perithecia, mushroom caps)
Major energy reserves used by spores	Mycolaminarin and lipids	Glycogen and trehalose, also sugar alcohols and lipid

1.4 Growing *Phytophthora in vitro*

There are several common natural medias used for the growth and identification of *Phytophthora*. The most common media used to culture *Phytophthora* on is V8 juice agar (Chen & Zentmyer, 1970). Potato dextrose agar, oatmeal agar and cornmeal agar are also recommended due to their high carbohydrate concentrations, making them excellent for mycelial growth; however they are not optimal media for sporulation as they often lack sterols (Erwin & Ribeiro, 1996). To generate spores, sterol and vitamin rich media such as the V8 juice, carrot, pea, bean seed and hemp seed media are optimal (Erwin & Ribeiro, 1996). For research purposes it would be desirable to culture *Phytophthora* using defined media in order to standardise procedures and increase reproducibility; however for most *Phytophthora* an defined medium is still unknown. Furthermore, the conditions and compounds required for culturing *Phytophthora* can vary greatly between the species; this means there is no one method for culturing *Phytophthora in vitro* (Erwin & Ribeiro, 1996).

Producing sporangia and zoospores *in vitro* is often a complex process which requires a series of specific environmental conditions (Erwin and Ribeiro, 1996). For most *Phytophthora* species sporangium production *in vitro* requires mycelium to be grown in a plant-based liquid medium (V8 juice, carrot or pea broth) followed by a period of starvation which involves washing and incubating the mycelia with salt solution or organic based solutions (for example soil extract or pond) to remove the nutrients (Zentmyer and Chen, 1969; Erwin and Ribeiro, 1996). The mycelia are incubated in the nutrient-deprived media generally under light, as light exposure is a common stimulus for generating sporangia (Harnish, 1965; Lilly, 1966; Brasier, 1969).

1.5 Kauri Dieback Disease

1.5.1 Kauri (*Agathis australis*)

New Zealand kauri are native conifer trees and are endemic to the lowland rainforests of the northern North Island of New Zealand (Beever *et al.,* 2009). Kauri are the largest and

longest lived native tree species in New Zealand (Ahmed & Ogden, 1987). A kauri tree can reach a height of 30 – 60 m and the maximum age of a kauri is estimated to be 1700 years old (Allan, 1961; Hooker, 2011; Ahmed & Ogden, 1987). The largest known kauri in volume is Tāne-Mahuta (Lord of the Forest), found in Waipoua Forest with a height of 51.2 m and a trunk girth of 13.8 m.

Kauri play an important ecological role in the indigenous forests they inhabit. These trees act as carbon sinks, influence surrounding soil, affect the availability of nutrients, water and light, and host a variety of epiphyte species (Steward & Beveridge, 2010; Wyse & Burns, 2013; Singh *et al.*, 2017). All of these aspects influence the surrounding plant communities and affect the ecosystem of the forest, making kauri a key-stone species (Wyse & Burns, 2013).

Kauri hold cultural importance and are considered a taonga (treasured) species within Māori communities (Steward & Beveridge, 2010). Kauri also generate commercial value by attracting a large number of tourists each year. People are attracted to the unique ecology of kauri forests and the impressive size of ancient kauri, like Tāne -Mahuta (Steward & Beveridge, 2010).

Kauri were extensively logged for their high quality timber in the 19th century after New Zealand was colonised by Europeans. Vast amounts of kauri forest were also cleared for farming, thus completely changing the upper North Island's forest landscape (Steward & Beveridge, 2010). The logging of kauri dramatically reduced the kauri population and now few mature kauri stand. It is estimated that since European colonisation, less than 1% of the original kauri forests remain (Steward& Beveridge, 2010). Those that do remain are mainly scattered across the Coromandel Peninsula, Great Barrier Island, Auckland and Northland regions, resulting in a low and fragmented genetic diversity (Wyse & Burns, 2013). In 1987, the New Zealand government declared all remaining kauri to be protected. The kauri that remain are now under threat of extinction from kauri dieback disease, caused by *P. agathidicida*.

1.5.2 *P. agathidicida* Causes Kauri Dieback Disease

Kauri dieback disease was first reported at Whangaparapara on Great Barrier Island in 1970 but was originally thought to be caused by *Phytophthora heveae* (Gadgil, 1974). In 2006 the disease became apparent in the Waitākere Ranges, where the same pathogen from Great Barrier Island was isolated from an unhealthy kauri (Waipara *et al.,* 2013). This pathogen was then identified as a new *Phytophthora* species, *Phytophthora* taxon Agathis (PTA; Beever *et al.,* 2009), which was then recently renamed and described as *P. agathidicida* (Weir, *et al.,* 2015).

P. agathidicida is still present in the Waitākere Ranges and has spread widely across to regional parks, plantations and private properties throughout Northland, Auckland and Great Barrier Island. The symptoms of the disease include canopy thinning, yellowing of foliage, root rot, "bleeding" of resins, death and localised extinctions (Beever *et al.,* 2009). These symptoms collectively are known as "kauri dieback". In addition to *P. agathidicida*, three other *Phytophthora* species have been found to be associated with kauri (*P. cinnamomi, P. multivora* and *P. cryptogea*); however these species are opportunistic pathogens that only cause death if the kauri's health is already compromised (Horner & Hough, 2014). *P. agathidicida* on the other hand has been shown to be aggressively pathogenic to kauri. The pathogen will infect kauri of all ages and can efficiently kill two year old kauri seedlings in only 20 days (Horner & Hough, 2013). *P. agathidicida* is host specific and it is hypothesised that *P. agathidicida* has a unique relationship with kauri; however there has been little research into the dynamics of this pathogenic relationship.

1.5.3 Kauri Dieback Management

P. agathidicida is thought to primarily be transmitted though mechanical vectors, mainly through human interaction on footwear (Pau'Uvale *et al*., 2010). It is also possibly transported by invasive mammals that are established in the ecosystems however evidence for this has yet to be proven (Pau'Uvale *et al*., 2010). The shallow kauri root systems are known to be easily disturbed by human and invasive mammals; this disturbance results in open wounds which provide an opportunity for infection to occur (Bellgard *et al.,* 2010). Therefore to control the spread of kauri dieback many walking tracks have been closed or

improved using board walks to reduce the stress on kauri root systems and to reduce the transfer of the pathogen into the soil. Public awareness campaigns have been put in place to educate the public on the disease. Foot washing stations have also been implemented at the start and end of walking tracks located in kauri forests (Pau'Uvale *et al*., 2010). The aims of these stations are for the public to remove dirt from footwear which could contain *P. agathidicida* and to wash the footwear with TriGene® (2%) to kill the pathogen before entering and leaving a kauri forest to reduce the spread of the disease (Pau'Uvale *et al*., 2010; Bellgard *et al.,* 2010). While this should be an effective method to reduce the spread of the pathogen, this process is heavily dependent on public compliance. A survey in Auckland in 2016 recorded more than 70% of the public were aware of kauri dieback disease. However, this did not translate into action as up to 83% of walkers using the tracks in the Waitākere Ranges were not using the cleaning stations (Singh *et al.,* 2017) and a substantial amount were reported to also walk off-track or disregarded closed tracks (Waipara, N., personal communication, December 11th, 2017).

Currently there are trials involving injecting phosphite into infected kauri to stop the spread of the disease within the tree (Horner & Hough, 2013). Phosphite affects the growth of pathogens and also stimulates the plants immune response (Horner & Hough, 2013). However phosphite is predicted to only provide temporary protection for three to five years (Horner *et al.*, 2015). So far the phosphite trials on kauri have stopped the infected trees from dying or decreasing in health. However these trees are not cured of the disease and the longevity of the treatments efficiency is uncertain (Horner *et al.*, 2015; Horner, I., personal communication, December 11th, 2017). As it stands there is still no cure for kauri dieback, so once a tree is infected, it will die. Thus a more stringent control of *P. agathidicida* is still needed for the protection of the kauri trees.

1.6 Addressing the Gaps in Knowledge

With *P. agathidicida* being an emerging pathogen, there is still much to learn about this species of *Phytophthora*. This thesis aimed to tackle some of these gaps in knowledge about the pathogen.

As of yet there is not an established protocol that generates large quantities of *P. agathidicida* zoospores *in vitro*. Detailed protocols for growing *P. agathidicida in vitro* are available (Weir *et al.,* 2015) however these do not generate sufficient amounts of zoospores for experiments. There are also protocols available which describe ample zoospore production in other more well-studied *Phytophthora* species (Chen & Zentmyer, 1970; Bouwmeester & Govers, 2009) however the conditions required to generate sporangia vary greatly between species. Therefore the first aim of this thesis was to develop an efficient method for producing *P. agathidicida* zoospores *in vitro* so zoospore experiments can proceed.

One area of interest which has not been explored yet is the attraction of *P. agathidicida* zoospores to kauri. In nature *P. agathidicida* is only known to infect and kill kauri, making it a host-specific pathogen. Host specificity is found in other *Phytophthora* species such as *P. sojae* where it exhibits a strong attraction to the isoflavones exuded from soybean roots. The detection of these isoflavones is thought to cause *P. sojae* zoospores to swim towards and infect its host (Morris and Ward, 1992; Suo *et al.,* 2016). It is therefore hypothesised that there is also a unique chemical interaction between *P. agathidicida* zoospores and kauri which could explain why it only infects kauri. Therefore the second aim of this thesis was to explore this chemical interaction at a microbial and a molecular level.

The ultimate aim of research into *P. agathidicida* is to find a cure for kauri dieback. Discovering a treatment that can kill *P. agathidicida* at all stages of its life cycle, with minimal phytotoxic effect on kauri and the environment is paramount for saving kauri. Currently only some commonly used fungicides and disinfectants have been trialled on *P. agathidicida* (Bellgard *et al.,* 2010), however there are more potential inhibitors yet to be tested on *P. agathidicida.* Therefore the third aim was to develop a high throughput screen of inhibitory compounds that can stop *P. agathidicida* across multiple life stages.

Chapter 2

Methods and Materials

2.1 Materials

Chemicals, unless stated otherwise, were purchased from Sigma Aldrich (St. Louis, MO, USA). Water was deionized and filtered by Milli-Q Integral Water Purification System (EMD Millipore, Billerica, MA, USA). Solutions and media were sterilised by autoclaving (≤ 121°C, 100 kPa for 20 min), unless stated otherwise.

2.2 Isolates

All *P. agathidicida* isolates used were provided by the Healthy Trees, Healthy Future Research Programme, Scion, Rotorua, New Zealand (Studholme *et al.*, 2016). Unless stated otherwise all work on *P. agathidicida* was conducted on isolate NZFS 3770.

Table 2.1:Isolates studied. Main isolate studied in bold (Herewini, 2017).

Isolate	Species	Host	Location	Year Isolated
NZFS 3770	***P. agathidicida***	***Agathis australis***	**Great Barrier Island, New Zealand**	**2006**
NZFS 3813	*P. agathidicida*	*Agathis australis*	Coromandel, New Zealand	2014
NZFS 3815	*P. agathidicida*	*Agathis australis*	Coromandel, New Zealand	2014

2.3 Growth Media

2.3.1 Clarified V8 Broth and Agar

To prepare V8 broth, 100 mL of Original V8 Juice (Campbell's) was diluted with 150 mL of water and 1 g $CaCO_3$ was added to adjust the pH to approximately 7.0. The juice was clarified by centrifugation at 4,000 x *g* for 10 minutes and passing the supernatant through a Whatman filter (Grade 1). Water was added to the filtered juice to make up 500 mL, and this clarified V8 (cV8) was diluted with water to make a 20% (v/v) broth, and sterilised by autoclaving (Jeffers, 2006).

To make cV8-agar, the cV8 was diluted to 20% (v/v) and agar was added to 1.5% (w/v) and sterilised by autoclaving. Petri dishes were filled with approximately 25 mL of molten agar and allowed to solidify. Once solidified, plates were stored at 4°C.

2.3.2 *Phytophthora*-selective Agar (PARP Agar)

PARP agar plates were prepared by mixing 17 g L^{-1} of pre-formulated cornmeal agar. Media were autoclaved and cooled to approximately 55°C before adding the antibiotics and fungicides (Table 2.2). Petri dishes were filled with approximately 25 mL of molten agar using aseptic techniques. Once solidified, the agar plates were stored in the dark at 4°C (Jeffers & Marin, 1986; Ferguson & Jeffers, 1999).

Table 2.2:Components of PARP Agar

Component	Weight/Volume per litre	Final Concentration (µg mL^{-1})
Cornmeal Agar	17 g	-
Ampicillin (500 mg mL^{-1})	500 µL	250
Rifampicin (20 mg mL^{-1})	500 µL	10
Pimaricin (2.5%)	400 µL	10
Pentachloronitrobenzene (40 mg mL^{-1})	2.5 mL	100

2.3.3 Carrot Broth

For 500 mL of carrot broth, 50 g of defrosted frozen carrots were blended with 200 mL of water for 4 x 30 second intervals. The blended carrots were filtered through 4 layers of cheesecloth and then through a Whatman filter (Grade 1). Water was added to make up a 500 mL solution which was sterilised by autoclaving. For the use of growing mycelial in liquid broth, the carrot broth was diluted to 10% (v/v).

2.4 Routine Culturing

P. agathidicida was routinely cultured on clarified 20% (v/v) cV8 agar. A plug of agar (~5 mm) containing *P. agathidicida* mycelium was placed centrally on a cV8 agar plate (standard Petri dish 90 mm diameter). After 5-7 days incubating at 24°C in darkness, a plug

of agar was removed from the leading edge of the mycelial growth and placed on a new cV8 agar plate; this culturing process was repeated 6-8 times. After culturing on agar 6-8 times, it is standard practice for *Phytophthora* cultures to passage through plant tissue to preserve the pathogenicity (Erwin & Ribeiro, 1996). For *P. agathidicida*, a plug of agar from the leading edge of the mycelial growth was passaged through plant material (*i.e.* a pear) according to standard protocols (Erwin & Ribeiro, 1996). Briefly, the plug of mycelium was placed in a pear and incubated for approximately 7 days at 24°C. A small section (5 mm diameter) from the pear was then cut from the advancing section of rot and placed on selective PARP agar to re-isolate *P. agathidicida* (Jeffers & Marin, 1986). The plates were incubated at 24°C for 5-7 days. Once *P. agathidicida* mycelium had grown on PARP, the incubation on cV8 agar restarted and the culturing cycle continued.

2.5 Zoospore Production

To produce sporangia and zoospores, plugs of agar containing *P. agathidicida* mycelium were grown in nutrient rich media which caused the mycelium to grow out from agar plug and thus were referred to as mycelial mats (Erwin & Ribeiro, 1996). Eight plugs (~6.4 mm diameter) of agar were taken from actively growing mycelium grown on cV8 agar and placed in 15 mL of diluted carrot broth (10% v/v). The plates were incubated over-night at 24°C in darkness.

The following day the carrot broth was decanted by pipetting and replaced with 15 mL soil extract (1% (w/v) in summer and 5% (w/v) in winter), enough to submerge the mycelial mats. To prepare the soil extract topsoil was collected native forest floor in Ross Creek, Dunedin, New Zealand. During the summer months, 20 g of the soil was mixed with 1 L of water, and in winter months, 50 g of the soil was mixed with 1 L of water. The soil was mixed for 4-6 hours using a magnetic stirrer and left to settle overnight. The next day the soil solution was centrifuged at 8,000 x *g* for 10 minutes, and filtered through Whatman filter paper (Grade 1). The soil extracts were sterilised by autoclaving, unless stated otherwise.

The soil extract was then decanted by pipetting and replaced at 30 minutes intervals, four times. After the last wash the mats remained in the soil extract and were placed under a 40 watt light, suspended 400 mm above the mats and left for 16 hours at 24°C.

After 16 hours of incubation with light, sporangia will have formed. To induce zoospore release the soil extract was then removed and replaced with 20 mL of cold sterile water and then incubated at 4°C for 30 minutes. Thereafter, the plates were brought back to room temperature. Large amounts of zoospore release typically began within 30 minutes of being at room temperature and zoospores were collected at 1 hour of being at room temperature to be used for chemotaxis, motility and germination assays.

2.5.1 Quantification of Sporangia

Optimising *P. agathidicida* zoospore production largely involved increasing the number of sporangia produced. During the initial attempts to optimise zoospore production a Nikon C-DS dissecting microscope was used at x40 magnification (the maximum available); the magnification was not enough to accurately visualise and count zoospores. However, it was sufficient to visualise the larger sporangia. Therefore the number of empty sporangia was initially used as a proxy to determine the success of zoospore production. For these experiments, three mycelial mats per Petri dish were chosen at random and the number of empty sporangia in the mycelial mats was counted and a mean was taken. For later experiments, an Olympus CKX41 inverted microscope was available, and quantification of zoospores proceeded as described below.

2.5.2 Quantification of Zoospores

To quantify the number of zoospores released, 5 mL of zoospore suspension was transferred to a 50 mm diameter Petri dish with a grid marked with 5 mm by 5 mm squares on the bottom. Once the zoospores encysted (typically 3-6 hours), the number of zoospores within a grid square was counted using an Olympus CKX41 inverted microscope at 40 x magnification. Three square grids were chosen at random and an average number of zoospores was calculated and multiplied to obtain the number of zoospores per mL. Zoospores were quantified before motility assays (2.8), germination assays (2.9) and

chemotaxis assays (2.10), as well as during optimisation experiments to assess zoospore production.

2.6 High Throughput Screening for Mycelial Growth Inhibition

The compounds used for the high throughput screen came from five 96-well Phenotype Microarray plates (Biolg Inc, California, USA). Each plate consists of different sets of chemicals preloaded and dried into wells of 96-well microplates. Plates PM21D-25D each contain 24 different compounds at 4 different concentrations. These compounds included antibiotics, antimicrobial metals, detergents and other known anti-yeast and antifungal chemicals (Appendix I). The compounds were resuspended by adding 20 µL of sterile water to each well. For screening, 5 µL of the resuspended compound was pipetted on to sterile filter paper disks (Whatman Grade 1, ~6 mm in diameter) and dried under sterile conditions for 30 minutes at room temperature. The four filter paper disks of each compound were arranged evenly on Potato Dextrose Agar (PDA; pre-formulated 39 g L^{-1}) with increasing concentration of the compound going clockwise; a control filter paper disk containing sterile water was also included (Figure 2.1). The plates were then inoculated with a 5 mm diameter agar plug of *P. agathidicida* mycelium, taken from the leading edge of an actively growing mycelial mat.

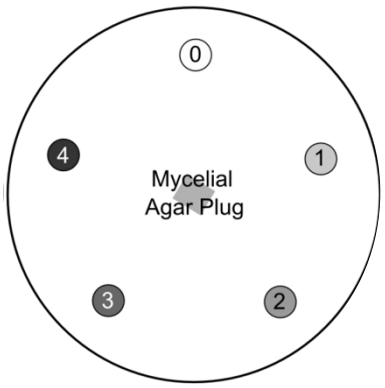

Figure 2.1: High-throughput screening for anti-oomycete compounds. A diagram showing the standard test arrangement. PDA is inoculated with an agar plug of *P. agathidicida* mycelium. Around the agar plug are disks saturated either with water (control; marked 0) or increasing concentrations of a test compound (in a clockwise direction, marked 1–4).

A control plate was also set up on PDA with just a control filter paper disc and a 5 mm agar plug of *P. agathidicida*. The plates were incubated in the dark at 22°C until the growth circle of *P. agathidicida* grew past the filter paper on the control plates. Three biological replicates for each compound were set up using independently grown *P. agathidicida* 3770 cultures. Any compound that reproducibly caused a zone of inhibition around the filter paper or any compound that reproducibly caused the mycelium growth rate to be less than the control, was deemed to be inhibitory.

2.7 Mycelial Growth EC_{50} Assays

Once an inhibitory compound was identified from the screen, an EC_{50} value was calculated for the mycelial growth of *P. agathidicida*. This was determined by measuring and comparing the radial growth of *P. agathidicida* mycelium growing on cornmeal agar amended with eight twofold dilutions of the inhibitory compound and a control plate (cornmeal agar only). Concentrations of the inhibitory compounds ranged from 0.125 to 512 μg mL^{-1}. For compounds which caused inhibition at low concentrations, a narrower range was used to get an accurate EC_{50} value. Three biological replicates for each compound were performed using independently grown *P. agathidicida* mycelium. A 5 mm diameter agar plug of *P. agathidicida* mycelium, taken from the leading edge of an actively growing mycelial mat, was placed in the centre of each test and control plate. The plates were incubated at 22°C in darkness for 7 days. The average radial growth of the mycelium from control and test plates was calculated by averaging the radius of the mycelial growth at four perpendicular points which intersect in the middle of the plate. The radial growth of each concentration and compound plate was divided by the average control plate and converted into the percentage of growth compared with control. To calculate the EC_{50} values, compound concentrations were log-transformed, and a normalised non-linear regression with curve fitting (by least squares) was carried out using GraphPad Prism version 6.0.

2.8 Motility Assays

To assess the effect of the anti-oomycete compounds on zoospore motility, 1 mL of zoospore suspension (1-5 x 10^4 zoospores per mL) was added to triplicate wells in a 24-well plate. Test compounds were added with concentration ranges from 0.125 to 512 μg mL^{-1}. Control wells were amended with equal volumes of sterile water (negative control) or 100 μg mL^{-1} benzalkonium chloride (positive control). Zoospores were kept at room temperature, and observed with an Olympus CKX41 inverted microscope to determine the time required for all zoospore motility to stop. Observations were made 5 minutes post-treatment, the minimum concentration value that stopped zoospore motility at 5 minutes was compared between the anti-oomycete compounds.

2.9 Germination Assays

To assess the effect of the anti-oomycete compounds on zoospore germination, 20 μL of zoospore suspension (1-5 x 10^4 zoospores per mL) was spread on 1 mL of amended water agar in a 24-well plate. Concentrations of the compounds ranged from 0.125 to 512 μg mL^{-1} and were repeated three times. Each 24-well plate contained a negative control (water) and a positive control (100 μg mL^{-1} Benzalkonium Chloride), both of which were also repeated in triplicate. The plates were incubated in the dark at room temperature for 12-16 hours, and the number of germinated and non-germinated zoospores per well were counted under the dissecting microscope at 40× magnification. Germination rates were calculated by dividing the number of germinated zoospores by the total number of zoospores in each well. The average zoospore germination rate compared with the negative control (water) was then calculated. The positive control wells should contain no germinated zoospores; if germinated zoospores were present then they may have germinated prior to the assay and so would be subtracted from the number of zoospores germinated within that plate, this however rarely happened. Spores were defined as being germinated if the germ tube length was at least double the length of the spore diameter, as previously described (Liu *et al*., 2014).

2.10 Chemotaxis Assays

Zoospores were generated from the methods previously described (2.5). Zoospores were pooled together and 5 mL of the pooled zoospore suspension were pipetted into a 50 mm diameter Petri dish, with an average of 1-5 x 10^4 zoospores per mL. Chemotaxis assays were performed using 2 μL Drummond microcapillary tubes (Sigma Aldrich, St. Louis, MO, USA) filled with either distilled water (negative control), cV8 (positive control) or a test chemical. The three capillaries were immediately submerged to prevent air bubbles entering the capillary and were placed centrally with equal distance apart in the 5 mL zoospore suspension. If the capillary contents are chemoattractive, most zoospores will swim towards the capillaries within 15 minutes and will encyst in approximately 60 minutes (Halsall, 1975), therefore the zoospore numbers inside the capillaries were counted after a 30 minute incubation at room temperature with no disturbance. The number of zoospores in each capillary was counted using an Olympus CKX41 inverted microscope at 40× magnification. Three independent pools of zoospores were used for each chemical tested, giving three biological replicates for each chemical. The positive controls on average had a range of 200-400 zoospores in the capillary; if the positive control had low zoospore numbers then the experiment was repeated. The number of zoospores that swam into the test capillary was compared against the negative control capillary using a paired-sample *t*-test.

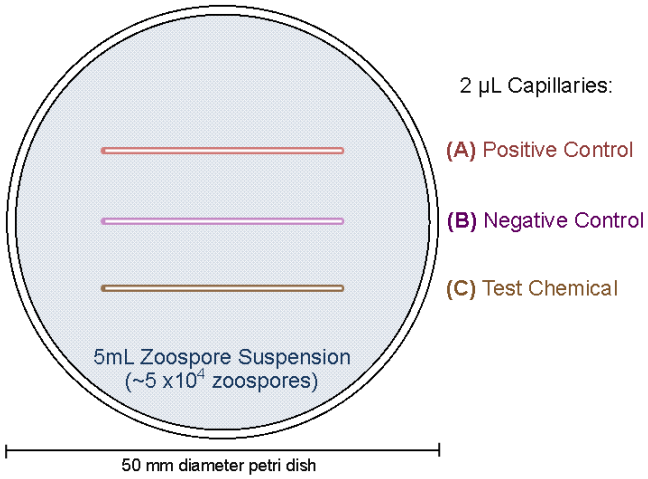

Figure 2.2: Chemotaxis Assay Schematic. The standard assay arrangement is shown. Three 2 µL micro-capillaries containing (A) the positive control, cV8 broth, (B) the negative control, water, and (C) the test chemical were placed in a 50 mm diameter Petri dish containing 5 mL of zoospore suspension.

2.10.1 Amino Acids and Sugars Chemotaxis Assays

A high (1 mM) and a low (50 µM) concentration of 19 L-amino acids and five sugars were dissolved in water and sterile filtered (0.45 µm). Chemotaxis assays which compared amino acids at different pH values had the amino acids (1 mM) adjusted to pH 3.0, 5.0, 9.0 and 11.0 using HCl and NaOH. The same amino acids were also dissolved in 10 mM phosphate buffer and adjusted to pH 7.0. Phosphate buffer (10 mM) was used as a negative control when using amino acids adjusted to pH 7.0.

2.11 Molecular Biology Techniques

All bacteria used for cloning and protein expression experiments were conducted with the strains presented in Table 2.3 and plasmids are described in Table 2.4.

Table 2.3: Bacterial strains used for cloning and protein expression

Strain	Purpose	Genotype	Source
E. coli BL21-Gold (DE3)	Protein expression	B F^- *ompT hsdS*(r_b^- m_B^-) *dmc*$^+$ Tc^R*gal* λ (DE3) *end*A Hte	Agilent
E. coli C41 (DE3)	Protein expression	F^- *ompT hsdSB* (*rB- mB-) gal dcm* (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Lucigen
E. coli Shuffle T7	Protein expression	*fhu*A2 *lac*Z:: T7 *gene1* [lon] *ompT ahpC gal λatt*::pNEB3-r1-c*DsbC* (Spec^R, *lacI*^{q}) *ΔtrxB sulA11 R(mcr-73::miniTn10*--Tet^S)2 [dcm] R(*zgb-210::Tn10* --Tet^S) *endA1 Δgor Δ(mcrC-mrr)114::IS10*	BioLabs
E. cloni 10G	General cloning strain	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *end*A1 *rec*A1 Φ80d*lac*ZΔM15 Δ*lac*X74 araD139 Δ(*ara,leu*)7697 *gal*U *gal*K *rps*L (Str^R) *nup*G λ - *ton*A	Lucigen

Table 2.4: Plasmids used for cloning and protein expression

Plasmid	Resistant marker	Use	Source
pET28	Kanamycin	Expression of *E. coli* tr-PIPK	Novagen
pGro7	Chloramphenicol	Expression ofGroEL/ES chaperone molecule to aid the expression of tr-PIPK	Takara
p426GPD	Ampicillin	Yeast-based reporter system	Addgene
p425GPD	Ampicillin	Yeast-based reporter system	Addgene

2.11.1 Media

For making broth, pre-formulated Luria-Bertani (LB; 25 g L^{-1}) was prepared in water and sterilised by autoclaving. For making agar, pre-formulated Luria-Bertani (LB; 25 g L^{-1}) and agar (12 g L^{-1}) was prepared in water and sterilised by autoclaving. Antibiotics were then added to the liquid agar after it had cooled to approximately 55^{o}C. Petri dishes were filled with approximately 25 mL of molten agar using aseptic technique. Once plates had solidified and dried, cells were applied via streaking and incubated at appropriate temperatures. The antibiotics used were kanamycin (30 µg mL^{-1}) and ampicillin (50 µg mL^{-1}).

2.11.2 Overnight Cell Cultures

Overnight cultures were typically 5 mL of the appropriate media with antibiotics if required. Cultures for protein expression experiments were grown in LB at 37^{o}C and aerated by shaking at 180 rpm. The media was inoculated using either freezer stock or from a single colony on an agar plate using sterile techniques.

2.11.3 Freezer Stocks

All bacterial cells used were permanently stored at -80^{o}C. Stocks were generated from overnight cultures mixed 1:1 with sterile 50% glycerol.

2.11.4 Electrocompetent Cells

Cultures of cells were grown in LB until the optical density (OD_{600}; optical density at 600 nm) reached 0.35-0.4. Cells were then chilled on ice for approximately 20 minutes before being centrifuged at 4^{o}C and 1700 x *g* for 15 minutes. The cells were washed in ice cold water and were again centrifuged at 4^{o}C and 1700 x *g* for 15 minutes; this was repeated 3-4 times. Cells were then resuspended in ice-cold 10% (w/v) glycerol and all cells were combined into a single 50 mL Falcon tube before being centrifuged at 4^{o}C and 1700 x *g* for 15 minutes. After centrifugation, the supernatant was discharged and cells were resuspended in residual liquid. Additional 10% (w/v) glycerol was added until the OD_{600} of a 1:1000 dilution of cells was ~0.1. Cells were then stored in 50 µL aliquots at -80^{o}C.

2.11.5 Transformation

Plasmid DNA was added to a thawed aliquot of electrocompetent cells using sterile techniques in a 2 mm MicroPulser electroporation cuvette (Bio-Rad, Hercules, CA, USA). The cells were electroporated with a 2.5 kV electric pulse for 3 ms in the BioRad MicroPulser (Bio-Rad), after which 500 µL SOC (super optimal broth with catabolite repression) media was added immediately. The cells were recovered for approximately 1 hour at 37^{o}C and then aliquots were spread on agar plates which were incubated overnight at 37^{o}C.

Successful transformations were confirmed with colony PCR and gene sequencing. For colony PCR a single colony was picked and mixed with 20 µL sterile water, 1µL of this suspension was used as the template in a standard PCR protocol with primers specific for the plasmids. The remainder of the cell suspension was used to inoculate a 5 mL LB culture including the appropriate antibiotic and incubated at 37^{o}C at 180 rpm overnight. If sequence of the plasmid is confirmed to be correct then the overnight culture was used to make a freezer stock. PCR products were confirmed via Sanger sequencing (Otago Genetic Analysis Services). Sequencing results were analysed with SnapGene software version 3.2.1.

2.12 DNA Techniques

2.12.1 Polymerase Chain Reaction (PCR)

PCR was used to generate fragments for cloning and for colony screening. The enzymes and reagents typically included Phusion polymerase (high fidelity polymerase used for accurate cloning, (BioLabs, New England, USA), dNTPs and reaction buffers (Table 2.5). Primers were designed using SnapGene software version 3.2.1 and ordered from Macrogen (Geumcheon-gu, Seoul, Korea).

The annealing temperatures and extension times were optimised for each reaction according to the calculated melting temperatures of the primers and the length of product to be amplified. The typical PCR conditions used are represented in table 2.5 and table 2.6. Reactions were conducted in an MJ Mini Thermocycler (Bio-Rad) using cycling conditions as shown in Table 2.7.

Table 2.5: Typical high fidelity PCR assay components.

Component	Volume
Nuclease-free water	31
5x GC Buffer	10
dNTPs (10 mM)	1
Forward Primer (10 µM)	2.5
Reverse Primer (10 µM)	2.5
DMSO	1.5
Template DNA (50 ng/µL)	1
Phusion Polymerase	0.5
Total	**50 µL**

Table 2.6: Typical two-step PCR reaction cycling conditions.

Temperature / °C	Time	
98	30 s	
98	10 s	
72	30 s	↰ Repeat x 30
72	5 m	
12	∞	

Table 2.7: Gene PCR primers used for the research described.

Primer Name	Sequence (5' – 3')	Purpose
GK4_Ala382_F	CAGCATATGGCGGTTAGCCAGACG CCG	Confirming the presence of tr-PIPK
GK4_R752_R	CAGTCTCGAGTTAACGACCGAAAATGACGGTATCAATCA	Confirming the presence of tr-PIPK
PTA_GK4_F	ATTGGATCCATGGCGGTGTGCGCGCCCGAGACGGC	To isolate and amplify GK4 into p426 & p425GDP
PTA_GK4_R	CGCAAGCTTCTATATCTCCATTGAGAGGTTGGAGTCCAATGAGACGTTCG	To isolate and amplify GK4 into p426 & p425GDP
PTA_GPR11_F	ATTGGATCCATGCAGCTGCAGACGCTGGCGTACGCGATC	To isolate and amplify GPR11 into p426 & p425GDP
PTA_GPR11_R	ATTAAGCTTTCAAAACATGTCGCCGCCAGGTGACGAGGAG	To isolate and amplify GPR11 into p426 & p425GDP

2.12.2 Plasmid Construction

Plasmids required for transformations were purified from overnight cultures using a Plasmid Mini Kit I (OMEGA Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. Sequence analyses were conducted using SnapGene software version 3.2.1 (GSL Biotech LLC).

Plasmid DNA and PCR products were digested using restriction endonucleases according to the manufacturer's instructions. Digest reactions typically contained 1 µg of DNA, 0.5 µL of enzyme at approximately 10 U, 1X ligation buffer, and were incubated at 37^{o}C for 1 hour. Reactions were terminated by heat-inactivation for 10 minutes at 65^{o}C. The digested products were visualised by gel electrophoresis and bands of interest were isolated using gel-extraction.

DNA fragments with cohesive ends were cloned into plasmids using ligation. Ligation reactions contained a 3:1 molar ratio of insert to plasmid DNA, 200 U of DNA ligase and 1X reaction buffer. Reactions were incubated at room temperature for at least 1 hour before being terminated by heat activation for 10 minutes at 65^{o}C. DNA was quantified via absorbance measurements at 260 nm in a BioPhotometer machine.

2.12.3 DNA Agarose Gel

DNA obtained from PCR was separated out according to size by gel electrophoresis. A 1% agarose gel was made with 1 x TAE buffer (400 mM tris-acetate pH 8.4, 1 mM EDTA, 20 mM acetic acid), containing 0.5 µg mL^{-1} ethidium bromide. DNA to be loaded onto the gel was mixed with loading dye (KAPA Biosynthesis), to reach a ratio of 1:6 of the dye and DNA. A DNA size marker, 1kb+ Universal Ladder (KAPA Biosystems), was loaded on the gel in parallel to samples. Gels were electrophoresed in 1 x TAE for 120 minutes at 110 V. Gels were visualised under a UV light in a Bio-Rad Gel Doc system.

For restriction digests on DNA fragments, DNA was separated in a gel containing SYBR Safe dye (Life Technologies). Bands in the gel were visualised using blue light trans-illumination and relevant bands were excised using a razor blade. DNA was purified with a Gel Extraction Kit (OMEGA Bio-Tek), according to the manufacturer's instructions.

2.13 Protein Techniques

2.13.1 Protein Expression Trials

The protein expression trials on tr-PIPK were conducted using 50 mL of LB in 200 mL Erlenmeyer flasks supplemented with kanamycin (30 µg mL^{-1}) and inoculated with an overnight culture of one of the expression strains (1:100 concentration). Flasks were incubated by shaking at 37°C until the cultures reached mid-log phase (OD_{600} ~0.5). Flasks were cooled on ice for 20 minutes prior to induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Following induction, cultures were shifted to an 18°C shaking incubator for 24 hours to overexpress the protein. One mL samples were collected at time points, 2 hours, 4 hours and 24 hours post-induction, as well as before induction (0 hour). The OD_{600} of these harvested cells the cells were pelleted by centrifugation 7000 x *g* for 10 minutes and stored at -20°C.

The pelleted cells were resuspended in lysis buffer (300 mM NaCl, 50 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.0) at 50 x the optical density in mL. Fifteen µL of the lysed cells was collected which corresponded to the total protein content of the cells collected. The remaining cells were centrifuged at 7000 x *g* and 15 µL of the supernatant was collected which corresponded to the soluble protein fractions. The representing fractions were analysed on SDS-PAGE (2.13.2; 2.13.3).

2.13.2 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualise and separate the proteins based on size. The components to make SDS-PAGE are shown in Table 2.8.

Table 2.8: SDS-PAGE recipe

Component	Resolving Gel (12%)	Stacking Gel (5%)
1.5 M Tris-HCl pH 8.8	5 mL	-
0.5 M Tris-HCl pH 6.8	-	2.5 mL
Water	8.7 mL	3.2 mL
SDS (10% (w/v))	200 µL	50 µL
Acrylamide/Bis (40%)	6 mL	500 µL
Ammonium persulfate (10% (w/v))	100 µL	25 µL
Tetramethylethylenediamide	20 µL	5 µL

The plates were aligned and held together by plastic in a bracket. The resolving gel was poured between the plates until the solution was approximately 2 cm from the top, then 70% (v/v) ethanol was overlain to prevent dehydration and to level the top of the resolving gels. After approximately 1 h, polymerisation of the gel would have occurred and the ethanol was rinsed off with distilled water. The stacking gel was then poured on top of the polymerised resolving gel and a comb was inserted into the liquid gel. Once polymerisation had taken place the gel was stored at 4^{o}C in damp paper towels to prevent dehydration from occurring.

2.13.3 Electrophoresis of Protein Samples on SDS-PAGE

Protein samples collected from protein expression trials were mixed at a 1:1 ratio with 2 x SDS-PAGE loading dye (100 mM Tris-Cl at pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 200 mM β-mercaptoethanol). The protein samples were then denatured by heating at 95^{o}C for 10 min. The SDS-PAGE gel was loaded with 5 µL of a molecular weight marker (Bio-Rad) and 10 µg of total and soluble protein fractions from the various time points. Electrophoresis was conducted in a Mini-Protean tetra cell system (Bio-Rad) at 200 V for 45 minutes in SDS-PAGE running buffer (25 mM Tris at pH 8.3, 250 mM glycine, 0.1% SDS). The separated proteins were visualised with Coomassie blue staining (1.5 M Coomassie R250, 50% methanol, 10% acetic acid) for approximately 30 minutes while gently rotating. The stain was then removed and the gel was transferred into destain (30% methanol, 10% acetic acid) until the gel was sufficiently de-stained and protein bands were clearly visible.

Chapter 3

Improving the Production of *P. agathidicida* Zoospores *in vitro*

3.1 Introduction

3.1.1 Uncertainty of Optimal Culture Conditions for *P. agathidicida*

Zoospores are infective motile spores which are dispersed from sporangia into the environment where they detect and infect their next host plant. Zoospores are fundamental to the spread of *Phytophthora* diseases and further research into their behaviour could be the substantial step in stopping the spread of the pathogens (Judelson, 2005). To conduct experiments on *Phytophthora* zoospores, adequate numbers of zoospores need be released *in vitro.*

The crux of producing enough zoospores for experiments is often forming sufficient amounts of sporangia. Sporangium production is a complex process and requires a series of specific environmental conditions to initiate the process (Erwin & Ribeiro, 1996). For most *Phytophthora* species sporangium production *in vitro* requires the *Phytophthora* mycelium to initially grow in nutrient rich liquid media (V8, carrot or pea broth), followed by washing and incubation in relatively nutrient deprived media (salt solutions, pond water or soil extract) to induce starvation (Zentmyer & Chen, 1969; Erwin & Ribeiro, 1996). The mycelial mats are incubated in the nutrient deprived media either with or without the presence of light (Harnish, 1965; Lilly, 1966; Brasier, 1969). Once sporangium has been produced, zoospores are often released when there is a drop in temperature (Erwin & Ribeiro, 1996).

Whilst there are many well established protocols for better studied *Phytophthora* such as *P. cinnamomi* (Chen & Zentmyer, 1970), the conditions needed to produce sporangium can vary greatly between *Phytophthora* species. Furthermore, generating enough zoospores *in vitro* for some *Phytophthora* species can be troublesome, so often optimisation of protocols is required (Erwin and Ribeiro, 1996).

P. agathidicida is an emerging pathogen and was only officially discovered to be the causative agent of kauri dieback in 2015 (Weir *et al,* 2015), therefore laboratory protocols which produce all parts of the lifecycle abundantly have not yet been described. A standard protocol from Weir *et al.* (2015) produced irregular and insufficient numbers of zoospores

for *P. agathidicida*. Therefore, the first aim of this research was to produce an improved and reliable protocol for producing *P. agathidicida* sporangia and zoospores so research could then be carried out on the zoospores. Temperature, light exposure, light intensity, time of incubations and media used for growth and washing are all variables that could affect the sporulation (Erwin and Ribeiro, 1996). These variables were examined here.

3.2 Results

3.2.1 The Effect of Light on Sporangium Production

Mycelial mats grown in 10% (v/v) carrot broth were incubated overnight in 5% soil solution and exposed to either darkness or a 40 watt light at 400 mm height (~2,000 $\mu W/cm^2$; Harnish, 1965). The next morning all treatments were washed in cold water to allow zoospore release from the sporangia and the number of empty sporangia was counted and compared between the light treatments (Figure 3.1). It was more reliable to count sporangia then zoospores because the resolution on the Nikon C-DS dissecting microscope was not strong enough to clearly visualise the zoospores, which are approximately 10 times smaller than sporangia (Erwin & Ribeiro, 1996). Furthermore, empty sporangia were chosen as a proxy because the clear empty spore was easily identified under the microscope and was not confused with oospores (Figure 3.1). Thus the number sporangia were used as a proxy for zoospore production success.

Figure 3.1: Difference in sporangial production after an overnight incubation with different light conditions. (A) Mycelia (shown by red arrow) before the 5% soil extract wash and light incubation. (B & C) Mycelial mats have been washed with 5% soil extract and exposed to different light conditions overnight and micrographs were taken 1 hour after the cold wash to allow for maximum zoospore release to take place. (B) Mycelia without light exposure, showing spherical oospores (red circle). (C) Mycelia exposed to light (40 watt light at 400 mm height), showing pear-shaped sporangia (red circle), after they had released zoospores. Micrographs taken at 40 x magnification on a Nikon C-DS dissecting microscope.

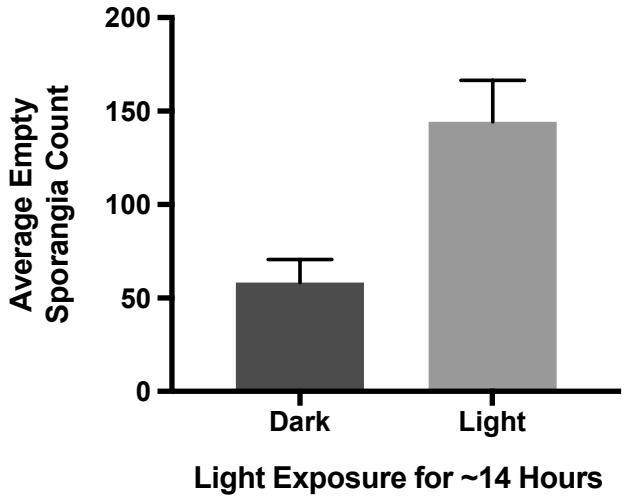

Figure 3.2: Sporangia counts after different light exposures. Mycelial mats washed with soil extract (5%) were exposed to either darkness or light (40 watt suspended 40 mm above the mats) over night. The number of empty sporangia in each petri dish was counted once zoospores were allowed to be released. A mean of empty sporangia from three dishes was calculated per light exposure. Bars represent the standard error of the mean.

There were significantly more sporangium production with a light stimulus then without (Figure 3.2), and thus more zoospores were released. Prior to the mycelial mats being washed with soil solution and exposed to a light treatment, the mycelium had no spores (Figure 3.1 A). Post-wash and light treatment resulted in an increase in spores in both dark and light treatments. Without a light stimulus the mycelial mats mainly produced oospores (Figure 3.1 B), whereas the light exposure mainly produced sporangia, which is shown by the large amount of empty pear-shaped spores (Figure 3.1 C). Therefore a 40 watt light at 400 mm height proved to be better at producing sporangia and thus zoospores during an overnight incubation.

The period of time exposed to a light stimulus was also important. A minimum of 8 hours was required to produce sporangia and a maximum incubation time was 14 hours. An incubation time longer than 14 hours would cause some sporangia to release zoospores before being exposed to low temperatures. Zoospores released before the cold washes cannot be used for experiments because they are still in the soil solution. Therefore the mycelial mats were incubated under a 40 watt light, suspended 400 mm above the mats for ~14 hours for maximum sporangial production.

3.2.2 The Effect of Washing Media on Sporangium Production

Based on preliminary studies conducted by Scion (personal communication), our initial protocol utilised pond water as the wash solution. Fresh pond water collected from Ross Creek, Dunedin during summer was initially used to wash the mycelial mats. However, changes in the weather and the seasons affected the pond water and caused a drop in sporangium production, therefore other options were explored. The following wash solutions were tested: Chen and Zentmyer salt solution (recommended synthetic washing solution for *P. cinnamomi*; Chen & Zentmyer, 1970), fresh pond water from Ross Creek (collected in March 2017) and soil extract at 1% or 2% (w/v) concentration (soil collected from Ross Creek in March 2017). The pond water and the soil extract solutions were either autoclaved or sterile filtered (0.22 µm). The mycelial mats were incubated under a light overnight and were exposed to low temperatures to induce zoospore release. Empty sporangia were counted once maximal zoospore release occurred ~1 hour after being at room temperature (Figure 3.3).

The salt solution and the sterile filtered (S.F) pond water produced no sporangia which released zoospores (Figure 3.4). The autoclaved (Auto) pond water collected at Ross Creek, Dunedin produced low numbers of sporangia (Figure 3.4). The soil extract produced the most sporangia and in return a lot of zoospores were released. The non-sterilised 1% soil extract produced the highest number of sporangia (Figure 3.4). Sterilisation of the 1% soil extract by either autoclaving or filtration greatly reduced the number of sporangia produced; however, autoclaved soil extract produced more spores than filtration (Figure 3.4).

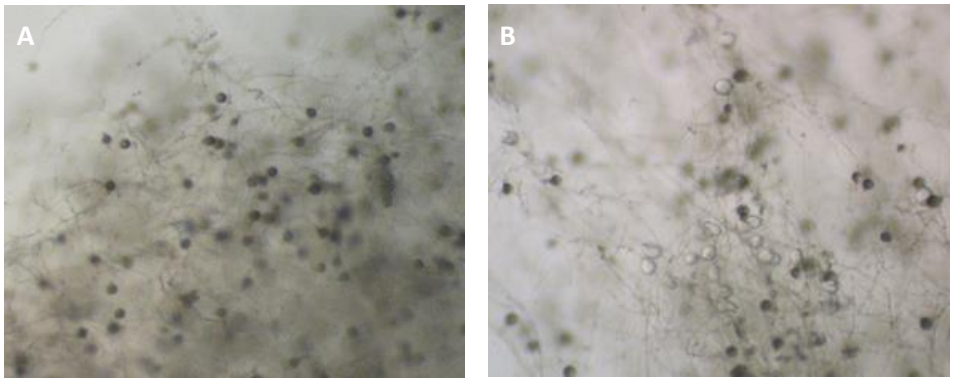

Figure 3.3: Mycelial mats after being washed with (A) pond water and (B) 1% sterile filtered soil extract. Micrographs taken at 10 x magnification on an Nikon C-DS dissecting microscope. Oospores are dark spheres, empty sporangia are slightly pear shaped and clear.

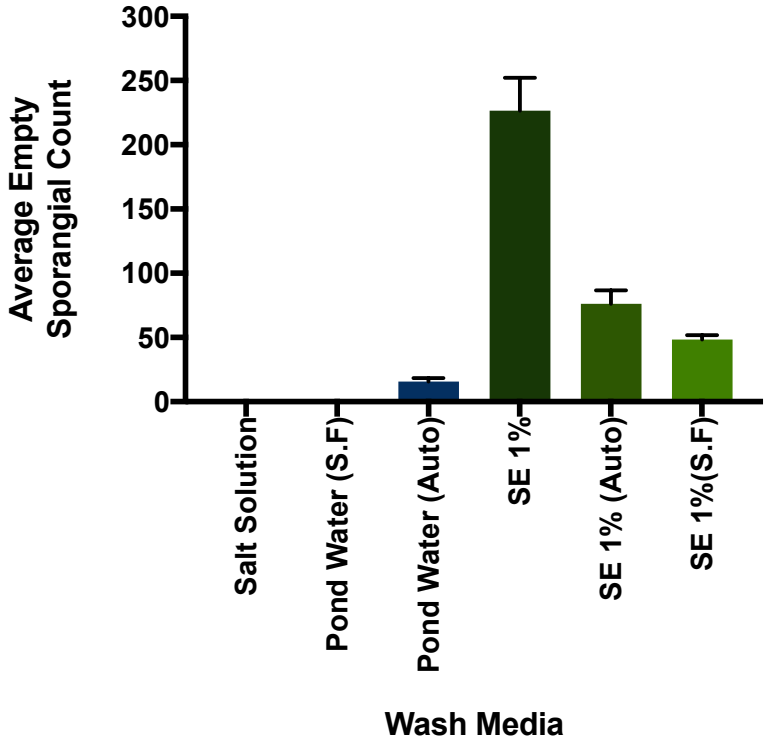

Figure 3.4: Empty sporangial counts after using different wash media. Petri dishes containing mycelial mats grown in carrot broth were washed with: Chen and Zentmyer Salt Solution, pond water collected from Ross Creek, Dunedin or soil extract at 1%. all washes were repeated on triplicate dishes. The number of empty sporangia were counted the next day once zoospores were allowed to be released. A mean sporangium count was calculated per wash treatment. Bars represent the standard error of the mean, n=3 (Auto = autoclaved, S.F = sterile filtered at 0.22 μm).

The number of sporangia, and thus zoospores, declined when soil extract solution was collected in winter. By increasing the concentration of soil extract to 5% (w/v) during winter the number of sporangia and zoospores increased relative to numbers produced from 1% (w/v) soil extract during summer (Figure 3.5).

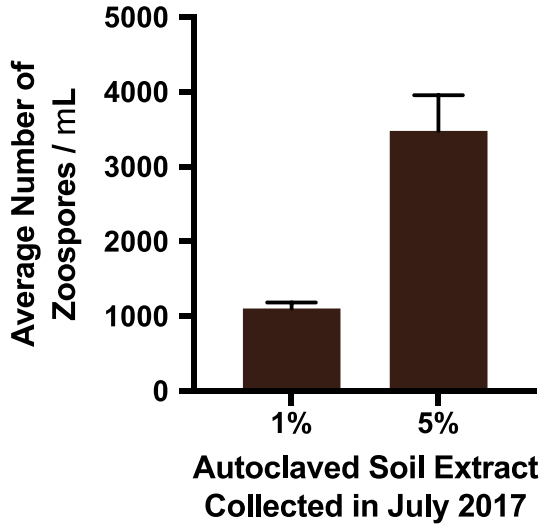

Figure 3.5: Zoospore numbers after washing with autoclaved soil extract collected in winter. Mycelial mats washed with soil extract collect in July 2017 at either 1% or 5% concentration. Mycelial mats in both treatments were grown in the same conditions. The number of zoospores released was counted using an Olympus CKX41 inverted microscope at 40 x magnification. Zoospore production using the 1% and 5% was repeated 3 times and a mean zoospore count was calculated per wash treatment. Bars represent the standard error of the mean.

3.2.3 Other Variables Tested to Increase Sporangium Production

A key part of most protocols for sporangium production is the growth of mycelial mats in liquid media. Carrot broth, pea broth and clarified V8 juice media are all common media for sporangium production for other *Phytophthora* species (Chen & Zentmyer 1970; Erwin & Ribeiro 1996). To test the optimal media for *P. agathidicida,* plugs of mycelium from cV8 agar were grown in sterile carrot broth, pea broth or clarified V8 juice media at either 100% or 10% (v/v) concentration, and then left at 24°C overnight in the dark. The resulting mycelial mats were then assessed for overall size increase from the plug of agar. The agar plugs grown in V8 media only had an average mycelial growth of ~3 mm, minus the diameter of the starting agar plug (~6 mm). The agar plugs grown in carrot broth and pea

broth had an average growth of mycelium of ~8 mm and ~7 mm respectively, minus the diameter of the starting agar plug (Figure 3.6).

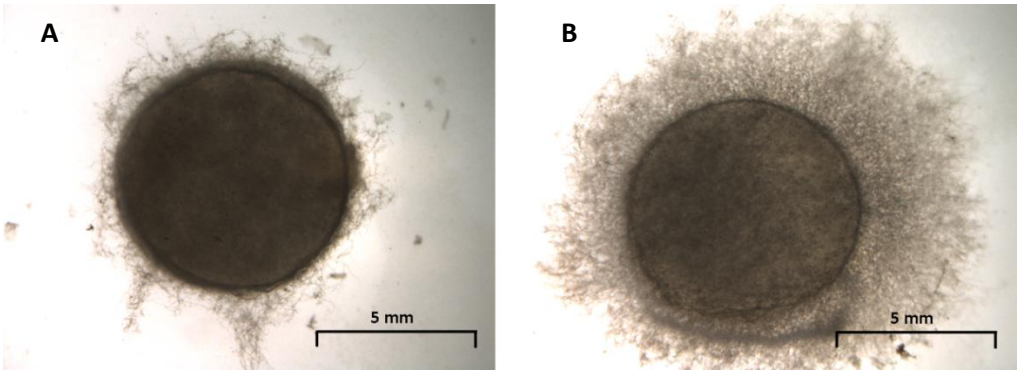

Figure 3.6: Mycelial mats after growing in (A) V8 Juice (10%) and (B) Carrot Broth (10%). Microscope photos taken at 4 x magnification on an Nikon C-DS dissecting microscope.

Optimal growth temperature is 21.5°C for *P. agathidicida* (Weir *et al*, 2015) however it also grew abundantly from 20-24°C, meaning a laboratory room temperature was often acceptable for growth as long as it did not drop below 20°C.

3.2.4 Passaging Through Kauri Leaves vs. Pear

It is standard practise for *Phytophthora* cultures to passage through plant tissue to preserve the pathogenicity and ability to produce zoospores (Erwin & Ribeiro, 1996). Passaging *Phytophthora* through a fleshy fruit (*i.e* pears) was recommended to increase zoospore production (Scion, personal communication). This was consistent with our observations that after roughly eight growth cycles on cV8 agar, the effectiveness of producing zoospores declined.

Passaging through a leaf of the host plant has also been reported to increase zoospore production (Bouwmeester & Govers, 2009). Both passaging techniques were therefore trialled on *P. agathidicida*. For the pear-passaging, an ~7 mm agar plug from actively growing mycelial mats was placed in a cut out section on pear and was incubated for 7 days at 24^{o}C. For the leaf-passaging, ~7 mm agar plugs from actively growing mycelial mats were placed on the centre of 3 fresh kauri leaves. After 5 days the agar plugs were removed from the leaves and the infected area of the leaves were cut out and

submerged in water, and then incubated at 24°C for 3 days. Once *P. agathidicida* had passaged through the pear and kauri leaves it was then grown on PARP agar to isolate *P. agathidicida* and was then transferred to cV8 agar. Once mycelia had grown on cV8 then the pear and leaf-passaged mycelia were subjected to normal zoospore production. Zoospore production was repeated 3 times using mycelial mats which had recently been pear or kauri leaf passaged or non-passaged, and the number of zoospores produced was counted and a mean value calculated.

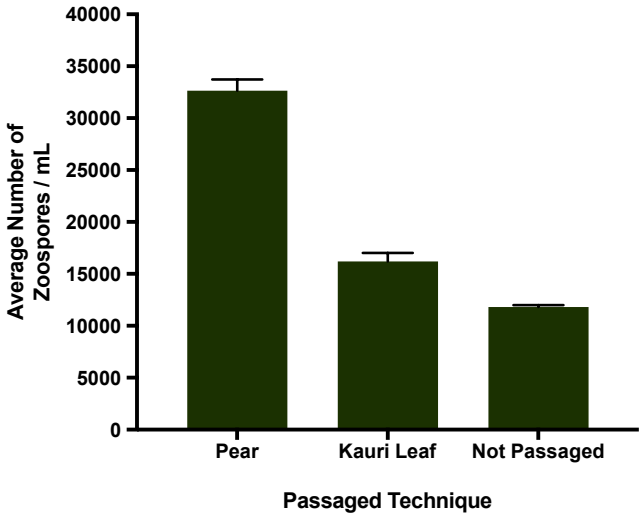

Figure 3.7: Zoospore counts after passaging through different passaging techniques. Each passage technique was repeated in triplicate and a mean zoospore count was calculated per passage treatment. The number of zoospores released was counted using an Olympus CKX41 inverted microscope at 40 x magnification. Bars represent the standard error of the mean.

Nearly twice as many zoospores were released after passaging through pear than kauri leaf. Thereafter, pear was then used to passage *P. agathidicida* (Figure 3.7).

An experiment was then set up to assess if passaging through pears altered the chemotactic ability of *P. agathidicida* zoospores. Zoospores were released from mycelium which grew on cV8 agar for one cycle after being passaged through a pear or a kauri leaf. These zoospores were used in a competitive chemotaxis assay. Zoospore suspensions (5 mL volume) from each passaging treatment were exposed to 2 µL capillaries containing kauri root extract, pine root extract and water. A similar number of zoospores were attracted to kauri and pine root extracts from both sources of zoospores (Figure 3.8). The control

capillaries with water both had no zoospores swim into them (not shown). These results verified that there was no difference in zoospore chemotactic behaviour between the two passaging techniques. Pear passaging therefore remained the preferred choice over kauri leaf passaging.

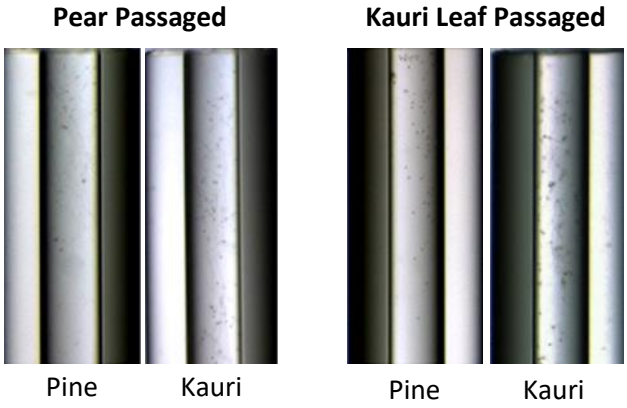

Figure 3.8: Chemotaxis assay of zoospores inside 2 µL capillaries after being produced from a pear or kauri leaf passage. The 2 µL capillaries were placed in 5 mL pools of zoospores that were released after being passaged through a pear or a kauri leaf. Micrographs taken at 40 x magnification on an Nikon C-DS dissecting microscope show the zoospores which swam into the capillaries containing either pine or kauri root extract.

3.3 Discussion

In this chapter an improved method of zoospore production was described for *P. agathidicida*. Based on the results of these experiments the optimal methods is to grow 6-10 discs (~6 mm diameter) of *P. agathidicida* mycelial mats in diluted carrot broth (10%) overnight. The following day these mycelial mats are washed with 5% soil extract and incubated in the soil extract for 14 hours under a 40 watt domed light. The mycelial mats are then washed in cold water and incubated at 4°C for 30 minutes to induce the sporangia to release zoospores. *P. agathidicida* was routinely cultured on cV8 agar and was passaged through a pear to increase fertility and pathogenicity every 8 passages. This method has continuously produced high numbers of zoospores which were able to be used for further research.

3.3.1 The Importance of the Wash Media in Sporangium Production

The wash medium was shown to be a key stimulus for sporangium production in *P. agathidicida* (Figures 3.3 and 3.4). In early *Phytophthora* experiments it was thought that organic solutions were the only way to generate sporangia *in vitro*, however it was discovered that distilled water with a specific combination of cations was also capable of producing sporangium (Chen & Zentmyer, 1970; Halsall, 1977). Chen and Zentmyer salt solution was generated from the four cations discovered to be essential for sporulation of *P. cinnamomi* and three other *Phytophthora* strains: Ca^{2+}, K^{+}, Mg^{2+}, and Fe^{3+}. The optimal concentration of these ions varies between *Phytophthora* species, but ranged between 0.1-1.0 mM. Using a synthetic salt solution, like Chen and Zentmyer salt solution, would have been the preferred wash medium for *P. agathidicida* to eliminate the changeability of organic wash solutions. Unfortunately *P. agathidicida* would not produce any sporangia with the Chen and Zentmyer salt solution (Figure 3.3), which lead to the use of autoclaved Ross Creek pond water as the initial wash solution.

Ross Creek pond water collected in summer 2016/17 produced sporangia. The chemical composition of the pond water was therefore sent for analysis to see why the pond water was a better wash solution than the Chen and Zentmyer salt solution. Interestingly chemicals in the pond water were cations similar to the cations in Chen and Zentmyer salt solution: $CaCO_3$ and dissolved Ca^{2+}, Mg^{2+} and K^{+} ions (Appendix II). The concentration of the cations was higher in the pond water than the synthetic salt solution. The success of the pond water could be because it has a higher concentration of these cations, however it is more likely due to the presence of more complex compounds which were not tested for in the chemical analysis.

From these results it is concluded that *P. agathidicida*, like many other *Phytophthora* species, will only respond to organic-based solutions (Erwin & Ribeiro, 1996). Seasonal and weather changes however have an effect on organic based media and thus affect the sporangium production. Ross Creek pond water became less effective at producing sporangium during summer 2017/18, which had extended cold and wet weather, and the pond water became completely ineffective during winter, hence the switch to soil extract. The 1% soil extract was also affected by seasonal changes, and reduced sporangium

production was seen during winter. By increasing the soil extract concentration to 5% sporangium production was restored to the levels obtained with soil collected in summer, making it the most reliable wash media. Sterilising the soil solution reduced the amount of sporangium produced. Interestingly there were also less sporangia produced when the soil extracts were sterile filtered than when they were autoclaved (Figure 3.4). The drop in sporangia during winter and when the medium was sterilised indicates that microbial activity within the soil may be a key component of stimulating sporangium production.

It has been reported that the presence of bacteria, such as *Chromobacterium* and *Pseudomonas,* stimulate the production of sporangia (Zentmyer, 1965; Chee & Newhook, 1966). There is evidence that there is a direct correlation between bacterial population within the soil extract and the number of sporangia produced (King, 1980), however the detailed relationship between the bacteria and *Phytophthora* is not well understood. In this experiment we observed that sterile filtering (0.22 µm) the soil extract produced less sporangia than autoclaving. This suggests that live bacteria are not essential but some heat stable microbial metabolite(s) produced from the cells could be involved in inducing sporangium production (Figure 3.4). Previous studies into the relationship between soil microbiota and *Phytophthora* have also come to the same conclusion (Marx & Haasis 1965; Zentmyer, 1965; Chee & Newhook, 1966; Ayers & Zentmyer, 1971); however no one has yet identified what these stimulating microbial metabolites could be. The chemical composition in the soil extract should also be analysed in future experiments to try and pin-point key compounds that initiate sporulation in *P. agathidicida*.

Another theory suggests that nonsterile soil extracts produces more sporangia, not because of specific interactions between the bacteria and *Phytophthora,* but because the bacteria and other microorganisms found in soil deplete the nutrient levels within the soil (Zentmyer & Chen, 1969; Ayers & Zentmyer, 1971). It is known that high nutrient levels, in particular high nitrogen and sugar levels inhibit sporangial production (Elliott, 1989).

3.3.2 Improving Sporangia Production (Other Variables)

Light was also shown to be a key stimulus for sporangium production for *P. agathidicida* (Figure 3.1). Light has been demonstrated before to be an important stimulus for producing

sporangia in other *Phytophthora* species (Harnish, 1965; Lilly, 1966; Alizadeh & Tsao, 1985). Airborne *Phytophthora* species tend to produce sporangia more efficiently under blue and near UV light (Ribeiro *et al.*, 1976) whereas many soilborne *Phytophthora* species produce sporangia in darkness (Erwin & Ribeiro, 1996). However for *P. agathidicida*, a soil-borne *Phytophthora* species, exposure to light was consistently shown to stimulate sporangial production (Figure 3.1). Interestingly, incubation in darkness for *P. agathidicida* caused low sporangial counts but high oospore counts (Figure 3.1 ad 3.2). Prior to washing the mycelium with low-nutrient media there are no spores formed within the hyphae and after the mycelium is washed sporulation occurs (Figure 3.2). It seems that the low-nutrient washing steps (section 3.2.2) induces sporulation to occur and the exposure of light seems to play a role in initiating sexual or asexual spore cycle in *P. agathidicida.* In the absence of light, more sexual oospores seemed to be generated and in the presence of light (~2,000 $\mu W/cm^2$) more asexual sporangia were generated (Figure 3.2).

Increasing mycelial growth in liquid cultures is important to get abundant numbers of sporangia but a larger mycelial mat does not guarantee more sporangia (Erwin & Ribeiro, 1996). As sporangia form on the leading edge of the mycelial mats, it is still important to grow larger mycelial mats so there is a larger surface area for sporangia to develop. Diluted carrot broth (10%) provided consistently good mycelial mats (Figure 3.6).

Long-term culturing on media is known to decrease pathogenicity of *Phytophthora.* It was noticed that *P. agathidicida* growing on cV8 agar after 8 or more passages began to produce fewer zoospores. It was therefore recommended to passage through a host plant or fleshy fruit to help replenish pathogenicity and zoospore numbers (Drenth & Sendall, 2004). Pear passaging was more successful than kauri leaf passaging (Table 3.1). A concern with passaging through pear is that this may change the behaviour of *P. agathidicida* as it does not grow in pear in nature. The chemotactic response of zoospores to kauri and non-kauri root extracts was analysed to assess if there were differences between the passaging. The same level of attraction to kauri was seen after being passaged through pears and kauri leaves, indicating that passaging does not affect the chemotaxis behaviour of *P. agathidicida* (Figure 3.8). This experiment was only conducted after a few months of

passaging; a longer term behavioural test should also be conducted to be able to conclude if passaging has an implication on *P. agathidicida* behaviour.

A factor not explored in this research is the use of sterols to develop sporangia. Sterols have been shown to increase asexual and sexual production *in vitro* in all *Phytophthora* species tested so far (Erwin & Ribeiro, 1996). *Phytophthora* require an exogenous source of sterols for sporulation as they lack a complete sterol synthesis pathway. It has been proposed that sterols play a key role in initiating asexual and sexual sporulation in *Phytophthora*, as sterols like β- hydroxysterols are important for membrane biogenesis of spores (Nes *et al.*, 1982). In nature, *Phytophthora* can source sterols potentially through their host plant, the requirement and recognition of sterols has been hypothesised to be an evolutionary adaptation of *Phytophthora* (Nes *et al.,* 1982). *In vitro*, sterols are found in most plant based media, or β-hydroxysterols, such as β-sitosterol can be added to media. Future experiments should analyse the soil extract for the presence of these sterols, to determine whether sterols are a key compound within the organic wash media, and supplement the wash media with sterols to see if this treatment affects *P. agathidicida* sporangium production. Knowing the chemical composition of the soil extract could mean a synthetic wash media could be generated.

At the beginning of this project, our zoospore release methods for *P. agathidicida* only produced ~100 zoospores per mL, whereas the protocol presented here produces 1-5 x 10^4 zoospores per mL. The refined method described in this chapter is also more reliable than previous methods; however occasionally we still have issues with producing adequate numbers of sporangia and zoospores, for no apparent reason. Interestingly, scientists who have been in the *Phytophthora* field for many years who are working on the most well studied *Phytophthora* species also occasionally encounter unpredictable sporangium and zoospore numbers (Erwin & Ribeiro, 1996). This is less of a reflection on the validity of the methods but more of a reflection of how little is truly known about the physiology of *Phytophthora* and the variability between species and strains.

Chapter 4

Exploring the Chemotactic Behaviour of *P. agathidicida* Zoospores

4.1 Introduction

4.1.1 Zoospore Chemotaxis

Chemotaxis is the directional movement of microbes towards a chemical gradient (Adler, 1966). Chemotaxis allows microorganisms to respond to their environment by swimming towards increasing concentrations of an attractant (chemoattraction) or swimming away from increasing concentrations of a repellent (chemorepulsion). Many microbial plant pathogens use chemotaxis to locate and/or colonise their host plant (Morris and Ward, 1992).

Phytophthora zoospores are known to exhibit chemotactic behaviour. Numerous species of *Phytophthora* have been shown to have a generalised chemotactic response towards plant exudates, such as sugars and amino acids (Dukes & Apple, 1961; Khew & Zentmyer, 1973; Suo *et al.,* 2016). In addition, some species of *Phytophthora* exhibit chemotaxis to host-specific plant chemicals. For example, *P. sojae* is strongly attracted to the isoflavones daidzein and genistein, which are exuded by the roots from legume plants, such as its host soybean (Morris & Ward, 1992). The survival of host specific pathogens, like *P. agathidicida* and *P. sojae,* relies on their ability to recognise specific host compounds and then move towards a host plant (Morris *et al.,* 1998).

P. agathidicida is so far only known to infect kauri in nature and therefore it is hypothesised that there is a specific signal interaction between *P. agathidicida* zoospores and kauri. The chemotaxis behaviour of *P. agathidicida* has not been explored before.

Chemotaxis behaviour of microbes can be assayed in the laboratory. These assays involve exposing microbes to a compound and observing whether or not the microbes swim towards the compound. The first aim was to use chemotaxis assays to build a repertoire of chemoattractants for *P. agathidicida*. Knowing what attracts *P. agathidicida* will help better understand the disease and could generate a new method to stop the spread of the disease.

4.1.2 *Phytophthora* Chemoreceptors

Eukaryotes possess a range of chemoreceptors called G-protein coupled receptors (GPCRs) that are responsive to external chemicals (Hua *et al.*, 2008). *Phytophthora* are known to have at least 50 GPCR genes (Bakthavatsalam *et al.*, 2006), where approximately 12 of these GPCRs are fused with a novel protein phosphatidylinositol phosphate kinase (PIPK) at the C-terminus (Bakthavatsalam *et al.*, 2006; Tyler *et al.*, 2007; Kay *et al.*, 2011). GPCR-PIPK genes only exist in two groups of eukaryotic organisms, *Dictyostelium spp.* and oomycetes (Rosenbaum *et al.*, 2009)

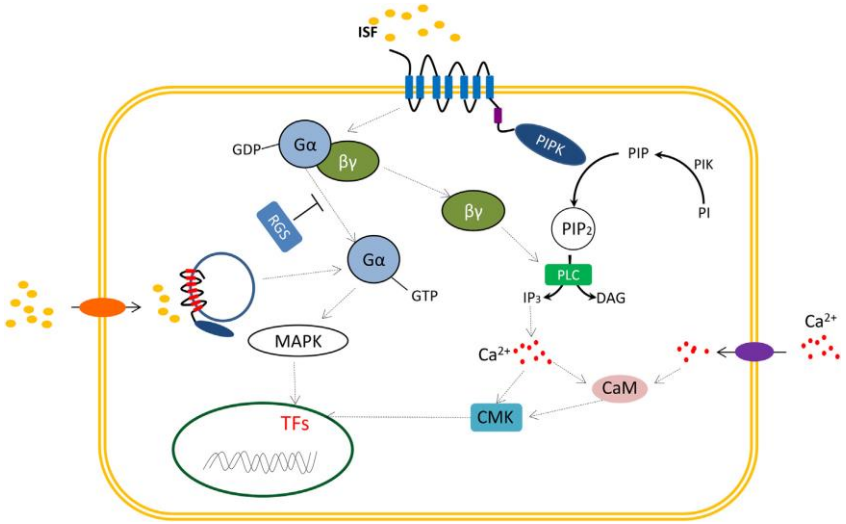

Figure 4.1: Proposed signal pathways involved in zoospore development and chemotaxis of *P. sojae*. ISF: isoflavones, RGS: regulator of G-protein signaling, PI: phosphoinositides, PIPK: phosphatidylinositol phosphate kinase, PLC: Phospholipase C, CaM: calmodulin, CMK: Ca^{2+} calmodulin binding kinase, TFs: transcription factors (Hua *et al*, 2015).

It is hypothesised that upon activation of a GPCR from an external signal, the associated PIPK converts phosphatidylinositol phosphate (PIP) to phosphatidylinositol bisphosphate (PIP2), which act as second messengers in a variety of pathways (Doughman *et al.*, 2003; Kwiatkowska, 2010). GPCRs and phospholipid signalling pathways have been shown to play an essential role in zoospore development, motility and virulence in *P. infestans* and *P. sojae* (Judelson & Roberts, 2002; Blanco & Judelson, 2005). Bioinformatic analysis of the

P. sojae genome resulted in the identification of the GCPRs: GK4 and GPR11, where GK4 is one of *Phytophthora's* GPCR-PIPK (Wang *et al.*, 2010; Yang *et al.*, 2013).

Silencing the GPCR genes *GK4* and *GPR11* caused reduction in zoospore development and pathogenicity in *P. sojae* (Wang *et al.*, 2010; Yang *et al.*, 2013). *GPR11* and *GK4* silenced transformants did not have impaired hyphal growth or morphology, or a reduction in sporulation or spore size. Silencing *GPR11* did however seriously disrupted zoospore release; of the silenced *GPR11* sporangium only half released zoospores, and zoospores that were released had a reduction in chemotaxis, encystment and germination rates (Wang *et al.*, 2010). *GK4* silencing also affected the chemotaxis behaviour of *P. sojae* zoospores, averting zoospores from swimming towards known attractants (isoflavones) or host plant extracts (Yang *et al.*, 2013).

The second aim was to take a molecular based approach towards understanding *P. agathidicida's* chemotaxis behaviour by researching into the PIPK protein and the ligand interactions with the GPCRs GK4 and GPR11. The first objective was to purify PIPK from *P. agathidicida* for future characterisation. The second objective was to construct a plasmid containing *P. agathidicida*'s *GK4* and *GPR11* genes which was to be used in a yeast-based reporter system in the future. A yeast-based reporter system is a technique which can be used as a screen to be able to identify ligand-GPCR interactions (Ladds *et al.*, 2003).

4.2 Results

4.2.1 Chemotactic Response of *P. agathidicida* Zoospores

The chemotactic behaviour of *P. agathidicida* was first assessed by testing its response to common primary metabolites released from roots into the soil, such as sugars and amino acids. All amino acids tested were L-isomers and have either been previously reported to be attractants of *Phytophthora* or have been reported to be present in root exudates of a variety of plants (Richter *et al.* 1968; Bertin *et al.*, 2003). According to the literature, different species of *Phytophthora* have preference for different amino acids and at a range of

concentrations (Khew and Zentmyer, 1970; Suo *et al.*, 2016), so both low and high concentrations of these compounds were tested (50 and 1000 µM, respectively). In each assay, the pool of zoospores contained a test chemical capillary and the matching negative (water) and positive (10% cV8) controls. The number of zoospores that swam into the test capillary was compared against the negative control capillary using a paired-sample *t*-test. Each compound was tested in triplicate using different pools of *P. agathidicida* zoospores. Any compound which was significantly different from the negative control ($p < 0.05$), was deemed to cause chemotaxis. To compare between the compounds, the counts were normalised by subtracting the number of zoospores that swam into the control (water) capillary from the number of zoospores in the test capillary.

4.2.1.1 Zoospore Chemotactic Response to Amino Acids

P. agathidicida zoospores were exposed to, alanine, arginine, asparagine, aspartate, cysteine, glutamine, glutamate, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine or valine at 50 or 1000 µM.

Of the non-polar amino acids tested, the aromatic amino acids, phenylalanine and tryptophan, produced the strongest chemotactic response (Figure 4.2). Phenylalanine caused strong chemoattraction at both concentrations, while tryptophan only caused significant chemoattraction at the higher concentration tested ($p < 0.05$ compared with the control). The other aromatic amino acid, tyrosine, did not cause a chemotaxis response from the zoospores.

Alanine, the simplest hydrophobic amino acid tested, also only caused some chemoattraction at 1000 µM, but this was not significant. The remaining hydrophobic amino acids, valine, methionine, leucine, glycine and proline did not produce a chemotaxis response from *P. agathidicida* (Figure 4.2).

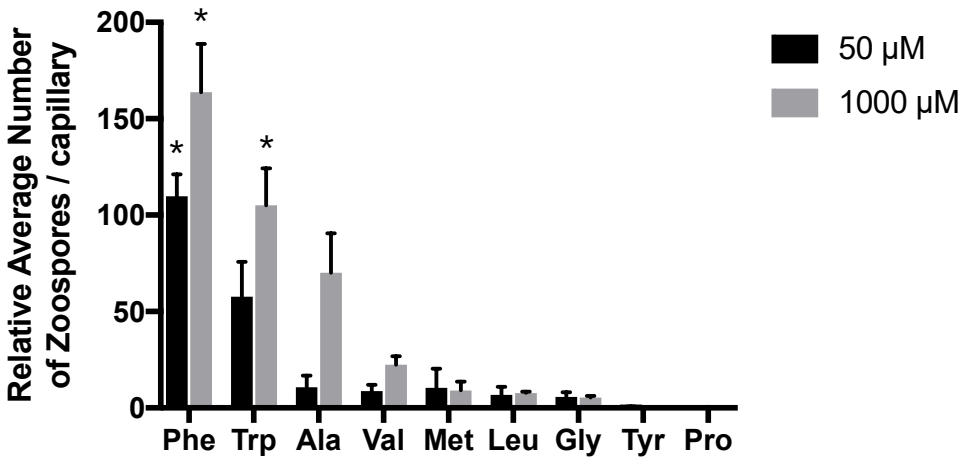

Figure 4.2: The chemotactic response of *P. agathidicida* zoospores towards non-polar amino acids. The number of zoospores which swam into a 2 µL capillary containing phenylalanine, tryptophan, alanine, valine, methionine, leucine, glycine, tyrosine or proline at 50 and 1000 µM were counted. The counts were normalised against the control capillary and a mean of three independent biological replicates was calculated. Error bars represent the standard error of the mean and the asterisks represent significant differences between the amino acid and the paired negative control before it was normalised: * = $p < 0.05$.

Zoospores were exposed to the polar amino acids serine, threonine, asparagine, glutamine or cysteine at 50 and 1000 µM. Asparagine, glutamine, threonine and serine all caused chemotaxis in *P. agathidicida* zoospores, cysteine however did not. Asparagine and glutamine caused strong chemoattraction of *P. agathidicida* zoospores at both concentrations ($p < 0.05$ compared with the control). Threonine also caused strong chemoattraction but only at 1000 µM ($p < 0.05$ compared with the control). Serine caused some chemoattraction but this was not significant when compared with the control.

Cysteine was the only polar amino acid not to cause chemotaxis in *P. agathidicida.* This could be because unlike the other polar amino acids, cysteine contains a thiol group.

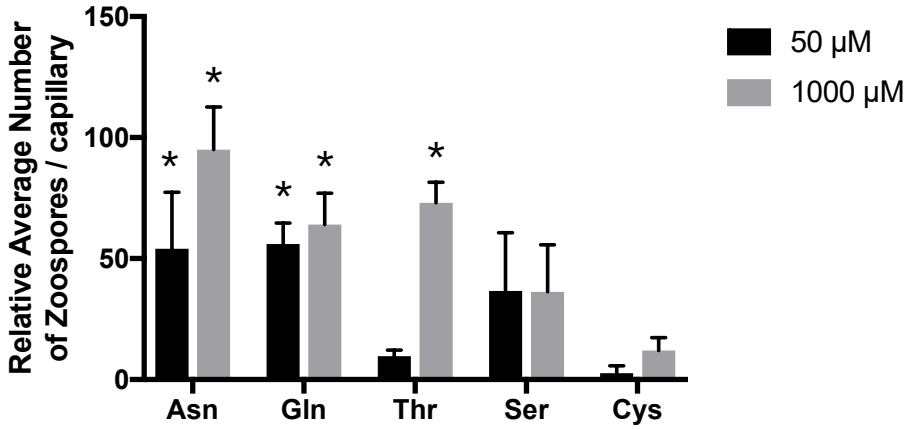

Figure 4.3: The chemotactic response of *P. agathidicida* zoospores towards polar amino acids. The number of zoospores which swam into a 2 µl capillary containing asparagine, glutamine, threonine, serine or cysteine at 50 and 1000 µM were counted. The counts were normalised against the control capillary and a mean of three independent biological replicates was calculated. Error bars represent the standard error of the mean and the asterisks represent significant differences between the amino acid and the paired negative control before it was normalised: * = $p < 0.05$.

P. agathidicida was exposed to the basic amino acids arginine, histidine and lysine at 50 and 1000 µM. Histidine, arginine and lysine did not attract *P. agathidicida* (Figure 4.4).

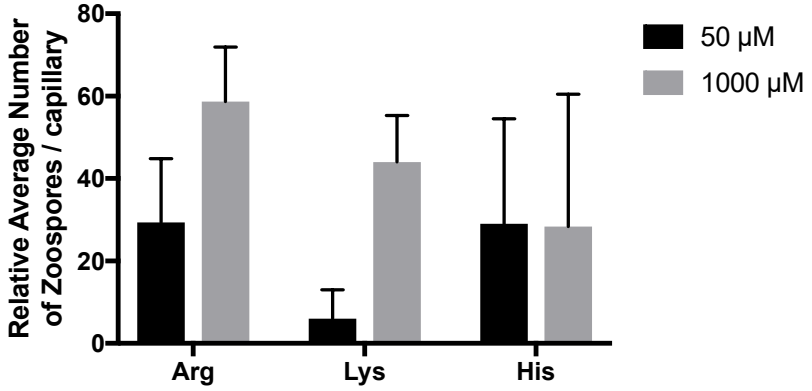

Figure 4.4: The chemotactic response of *P. agathidicida* zoospores towards basic amino acids. The number of zoospores which swam into a 2 µl capillary containing arginine, lysine or histidine at 50 and 1000 µM were counted. The counts were normalised against the control capillary and a mean of three independent biological replicates was calculated. Error bars represent the standard error of the mean and the asterisks represent significant differences between the amino acid and the paired negative control before it was normalised..

Both of the acidic amino acids, glutamate and aspartate, elicited strong chemoattraction at both concentrations (Figure 4.5; $p < 0.05$ compared with control).

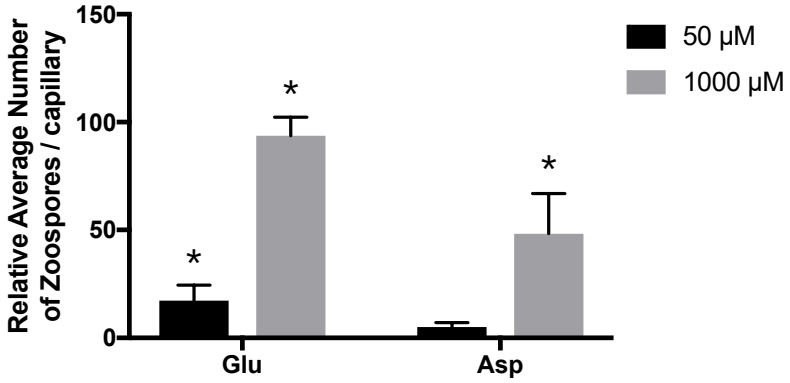

Figure 4.5: The chemotactic response of *P. agathidicida* zoospores towards acidic amino acids. The number of zoospores which swam into a 2 µl capillary containing glutamic acid or aspartic acid at 50 and 1000 µM were counted. The counts were normalised against the control capillary and a mean of three independent biological replicates was calculated. Error bars represent the standard error of the mean and the asterisks represent significant differences between the amino acid and the paired negative control before it was normalised: * = $p < 0.05$.

4.2.1.2 Zoospore Chemotactic Response to Sugars

Six different sugars were tested: glucose, fructose, lactose, galactose and sucrose at 50 and 1000 µM. Fructose and galactose were only strong attractants at 1 mM. Glucose, lactose and sucrose were strong attractants for *P. agathidicida* at both concentrations with sucrose being over 3 times more attractive.

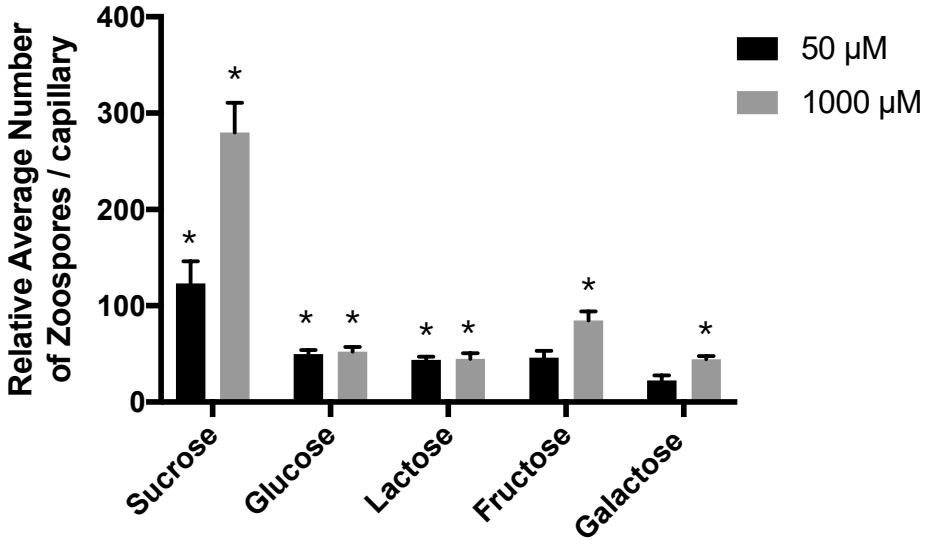

Figure 4.6: The chemotactic response of *P. agathidicida* zoospores towards sugars. The number of zoospores which swam into a 2 µl capillary containing sucrose, glucose, lactose, fructose or galactose at 50 and 1000 µM were counted. The counts were normalised against the control capillary and a mean of three independent biological replicates was calculated. Error bars represent the standard error of the mean and the asterisks represent significant differences between the amino acid and the paired negative control before it was normalised: * = $p < 0.05$.

4.2.2 Effects of pH on *P. agathidicida* Chemotaxis

The amino acids tested in 4.2.1 showed that the basic amino acids and most hydrophobic amino acids did not produce a chemotactic response but the acidic and most polar amino acids did. To test whether different ionic forms of the amino acids affects the chemotaxis behaviour of *P. agathidicida,* glutamate and arginine's pH were changed to 3.0, 5.0, 7.0, 9.0 and 11.0. Chemotaxis assays were set up as previously done with a water negative control, cV8 as a positive control and either glutamate or arginine at a certain pH level. Water controls with pH altered to 3.0, 5.0, 7.0, 9.0 or 11.0 were also tested separately to see if the addition or removal of hydrogen ions affected chemotaxis behaviour. The pH altered

water controls had little or no zoospore chemotaxis towards them (data not shown), it was therefore concluded that hydrogen ions did not bias any chemoattraction observed.

Glutamate was shown to be a strong chemoattractant in 4.2.1; its pH in water at 1 mM is approximately 3.5 and at pH 3.0 it was shown again to be a strong chemoattractant. For the remaining pH values however there was a dramatic drop in zoospores numbers with an increase in pH (Figure 4.7 A)

Arginine's unadjusted pH at 1 mM in water was approximately pH 11.0 and it was shown not to cause chemotaxis for *P. agathidicida* zoospores in experiment 4.2.1. However when arginine's pH was dropped to pH 3.0 it caused strong chemotaxis from *P. agathidicida* zoospores (Figure 4.5 B), but did not cause attraction at a higher pH.

This experiment showed *P. agathidicida* favours an acidic pH environment. A paired *t*-test was conducted between each pH value and the control; both glutamate and arginine had p values < 0.05 at pH 3 compared with the control and any other pH value.

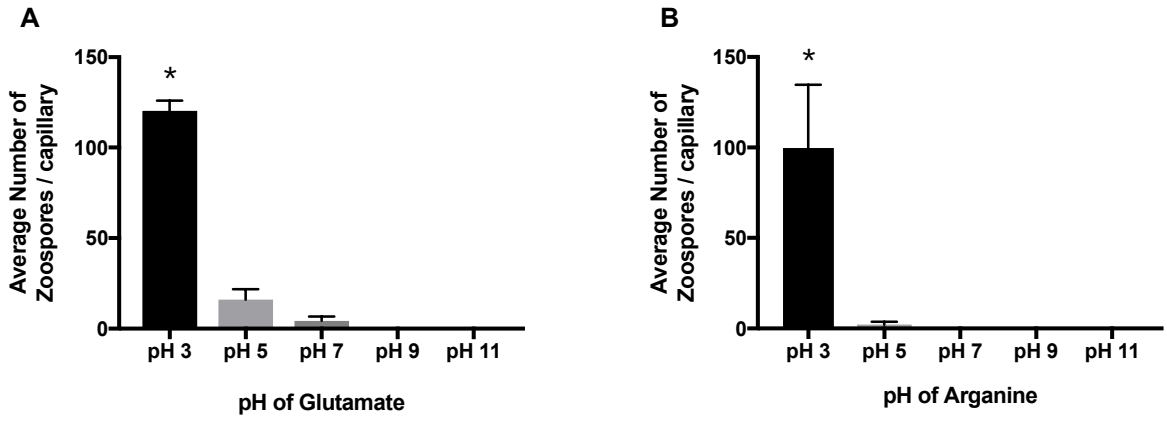

Figure 4.7: Chemotactic response of *P. agathidicida* zoospores to amino acids with adjusted pH values. The number of zoospores which swam into a 2 µl capillary containing 1000 µM of (A) glutamate or (B) arginine at pH 3, 5, 7, 9 or 12. The counts were normalised against the control capillary and a mean of three independent biological replicates was calculated. Error bars represent the standard error of the mean and the asterisks represent significant differences between the amino acid and the paired negative control before it was normalised: * = $p < 0.05$.

4.2.3 Germination Response of *P. agathidicida* to Amino Acids

The amino acids tested in 4.2.1 showed that the acidic amino acids and some polar amino acids produced a chemotaxis response from the zoospores but the basic amino acids did not. A brief experiment was then conducted to analyse the zoospore germination rates when exposed to different amino acids. Three biologically independent germination rates were calculated for each amino acid at either 50 or 1000 µM and the mean germination rate was calculated and made into a percentage of the control (Table 4.1).

Arginine, aspartate and asparagine all caused lower germination rates compared with the control (water) however the difference is not statistically significant (*t*-test, $p > 0.05$).

Table 4.1: Germination rates of *P. agathidicida* zoospores when exposed to amino acids. Germinated zoospores exposed to agar emended with arginine, aspartate or asparagine (50 and 1000 µM) were counted and recorded as a germination rate ($\frac{number of\ germinated\ zoospores}{total\ number\ of\ zoospores}$). Three biological replicates were averaged and calculated as a mean percentage of the control.

	Concentration of Amino Acid (µg/mL)	Germination Rate, Percentage of Control (%)
Water (Control)	0	100
Arginine	1000	70
	50	74
Aspartate	1000	61
	50	68
Asparagine	1000	80
	50	72

4.2.4 Inhibition of Zoospore Chemotaxis

GPCR-PIPK have been previously demonstrated to be involved in chemotaxis of other *Phytophthora* species. To probe the role of these receptors in *P. agathidicida*, a series of five commercially available PIPK inhibitors were tested against *P. agathidicida* zoospores. Zoospores were exposed to each inhibitor individually and were then subjected to a chemotaxis assay with a negative control (water) and a positive control (carrot broth). This was repeated in triplicate with different pools of zoospores each time. The results are shown in Table 4.2. Of the five inhibitors tested, three had no effect on zoospore chemotaxis: LY294002, LY30351 and Wortmannin. In contrast, the inhibitor Z5TK also reduced chemotaxis, while GSK2126458 prevented chemotaxis completely. Interestingly,

these zoospores still remained motile; however, they were unable to navigate towards the positive control.

**Table 4.2: PIPK inhibitors effect on *P. agathidicida's* ability to chemotaxis. ***statistically similar zoospore counts compared with the negative control

No Treatment	Average Number of Zoospores in Control Capillary
Negative Control (water)	0.3
Positive Control (carrot broth)	124.0
Zoospores Treated With PIPK Inhibitors (10 µM)	**Average Number of Zoospores in Positive Control Capillary**
GSK2126458	0.3*
Z5TK	28.3
LY294002	81.0
LY30351	86.7
Wortmannin	128.3

4.2.5 Heterologous Expression of the PIPK Domain in *Escherichia coli*

4.2.5.1 Cloning of the tr-PIPK Construct

Given that inhibiting PIPK impeded chemotaxis of *P. agathidicida* zoospores (4.2.4), molecular research into this unique protein ensued. The aim was to express and purify the protein for functional and structural studies. A PIPK, truncated from the GPCR (tr-PIPK), was inserted into the plasmid pET28, this was completed by an honours student, Alison McGhie. This plasmid encodes a His_6 tag at the C-terminus of the *tr*-PIPK fragment insert site to facilitate purification. Protein expression was from the T7 promoter under repression by the *lac* repressor molecule, and the plasmid encoded a kanamycin resistance cassette. The general cloning strain *E. coli* 10G was transformed with pET28-tr-PIPK plasmid. PCR and DNA sequencing confirmed cloning and the presence of the tr-PIPK insert in the construct (Figure 4.8).

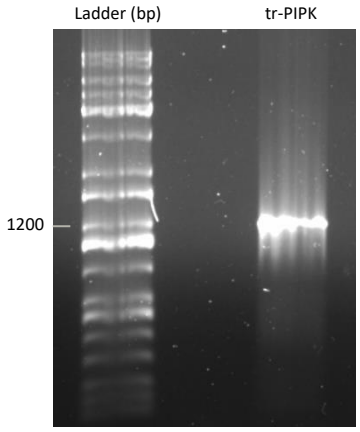

Figure 4.8: PCR amplification of tr-PIPK for confirmation. The reaction was separated on a 1% agarose gel containing ethidium bromide stain at 1 x concentration. The ladder was the KAPA 1kb Plus DNA ladder. The band in the ladder known to be 1200 base pairs (bp) is indicated. The tr-PIPK was amplified using gene specific primers (GK4_Ala382_F and GK4_R752_R) with a predicted product size of 1135 bp.

4.2.5.2 PIPK Expression Trials

The protein expression strain *E. coli* BL21-Gold (DE3) was transformed with the pET28-tr-PIPK plasmid. Isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM) was used to induce protein expression from the T7 promoter at the mid-log phase of growth. Samples were collected before induction (0h), and at 2, 4 and 21 hours post-induction, all samples were separated into total and soluble fractions. Coomassie blue staining and SDS-PAGE analysis revealed the presence of an overexpressed band in the total cell extracts near the expected molecular mass of the His-tagged tr-PIPK (43.8 kDa; Figure 4.9). However, no soluble PIPK was observed.

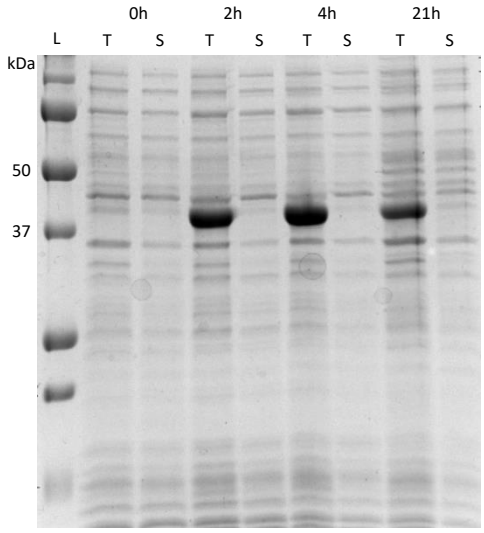

Figure 4.9: Expression trial of tr-PIPK. Cells were induced to express pET28-tr-PIPK at 28^{o}C. Total (T) and soluble (S) cell extracts were collected at indicated time points. Samples were visualised with Coomassie blue after separating through a 12% SDS-PAGE gel. Lane (L) was the molecular size marker (Bio-Rad precision plus) with known 37 and 50 kDa bands marked. Time points indicate when samples were collected after induction with IPTG, with 0h samples being collected before induction.

In an attempt to improve the solubility of the tr-PIPK domain three alternative *E. coli* protein expression strains were trialled: C41 (DE3); BL21-Gold (DE3) with pGro7; and BL21-Gold (DE3) Shuffle T7. Each strain has different properties that can improve protein expression. The strain C41 (DE3) has uncharacterised genomic mutations that improve expression of toxic proteins (Dumon-Seignovert *et al.*, 2004). The pGro7 plasmid encodes the GroEL and GroES molecular chaperones to assist in folding proteins (Ryabova *et al.*, 2013). The Shuffle T7 strain facilitates post-translational modifications, in particular producing disulphide bonded proteins (Lobstein *et al.*, 2012). The inductions were also conducted at a lower temperature (18^{o}C) to help improve solubility by limiting aggregation and increasing stability of the protein (Sørensen & Mortensen, 2005; Khow & Suntrarachun, 2012).

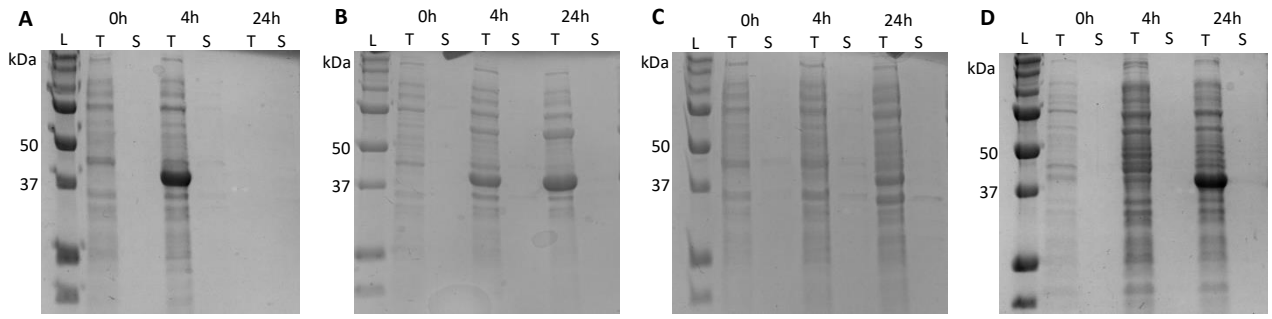

Figure 4.10: Expression trial of tr-PIPK with alternate *E. coli* strains. Different host strains were induced to express pET28-tr-PIPK at 18^{o}C. (A) BL21, (B) BL21(pGro7), (C) C41 and (D) Shuffle T7 in LB. Total (T) and soluble (S) cell extracts were collected at indicated time points. Samples were visualised with Coomassie blue after separating through a 12% SDS-PAGE gel. Lane (L) was the molecular sized marker (Bio-Rad precision plus) with known 37 and 50 kDa bands marked. Time points indicate when samples were collected after induction with IPTG (4, and 24 hours), with 0h samples being collected before induction.

The expression trials (Figure 4.10) indicate that all four strains were able to over express tr-PIPK at the predicted molecular mass (43.8 kDa) however only in the total fractions and again not in the soluble fractions. *E. coli* C41 (DE3) and Shuffle T7 cells did produce very small bands in the soluble fractions however not in enough quantity to perform purification.

Expression trials were then conducted using *E. coli* C41 (DE3) strains in 18^{o}C with alternative media, Luria-Bertani (LB), Terrific Broth (TB) and Auto Induction Media (AIM). Similar low amounts of tr-PIPK were expressed in the soluble fractions in LB, TB and AIM (data not shown).

4.2.6 Developing a Reporter System to Explore *Phytophthora* GPCR Ligand Binding

To begin to understand the chemotaxis behaviour of *P. agathidicida* at a molecular level, two GPCRs, GPR11 and GK4, were selected to be put through to a yeast-based reporter system (Ladds *et al.*, 2003). This system expresses exogenous GPCRs in engineered yeast which are set up to induce *lacZ* expression when the exogenous GPCR is activated by a ligand. Therefore this could be an innovative technique to screen *P. agathidicida* GPCRs against a library of ligands to determine what chemicals interact with these receptors. This system is run by collaborators in Cambridge University, UK (Ladds *et al.*, 2003). Plasmids containing the GPCRs, GPR11 and GK4 were therefore needed to be constructed. These

GPCRs were chosen because they have been shown to play an important part in *Phytophthora* chemotaxis and zoospore development before (Wang *et al.*, 2010; Yang *et al.*, 2013).

4.2.6.1 Construction of GK4 and GPR11 Reporter Plasmid

Genomic DNA was extracted form *P. agathidicida* mycelium. Four protocols from Promega Wizard Genomic DNA extraction kit were trialled. The plant tissue protocol was the only method to yield enough genomic DNA; 40-60 mg of ground mycelial mat yielded up to 120 ng μL^{-1} of genomic DNA using this method. The other protocols trialled had maximum yield of genomic DNA of only 7 ng μL^{-1}.

GK4 and *GPR11* genes were amplified from the genomic DNA of *P. agathidicida* using PCR and designed gene specific primers. The predicted size of the amplification product of *GK4* was 2424 bp, which is shown to be present by the single bright band in Figure 4.11(A) between the 3000 and 2000 bp bands. The predicted size of the amplification product of *GPR11* was 1044 bp, which is shown to be present by the single band in Figure 4.11(B) between the 1200 and 1000 bp bands.

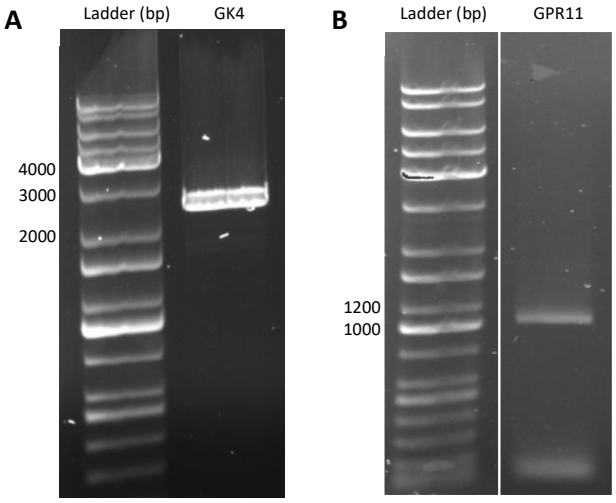

Figure 4.11: Amplification of *P. agathidicida* (A) *GK4* and (B) *GPR1* genes. PCR products of *GK4* and *GPR11*genes were separated on a 1% agarose gel containing ethidium bromide stain at 1 x concentration. The ladder was the KAPA 1kb Plus DNA ladder. The band in the ladder known to be 1000, 1200, 2000, 3000, 4000 base pairs (bp) is indicated. GK4 and GPR11 were amplified using gene specific primers with the predicted sizes to be 2424 and 1044 bp respectively.

The amplicons from the PCR were purified and were subjected to restriction digest with the restriction enzymes BamHI and HindIII. The plasmids p425GPD and p426GPD were also purified and were subjected to restriction digest with the restriction enzymes BamHI and HindIII. The fragments were then gel extracted from a SYBR Safe gel and purified ready for ligation and cloning. The digested products were ligated using T4 DNA ligase. Both fragments were separately ligated and then cloned into the vectors p425GPD (leu2 marker) and p426GPD (ura3 marker) in *E. cloni* 10G. The presence of the *GK4* and *GPR11* genes in the plasmids were confirmed by DNA sequencing.

The four successfully constructed plasmids, p425GPD-GK4, p425GPD-GPR11, p426GPD-GK4 and p426GPD-GPR11 were sent to Dr. Graham Ladds (University of Cambridge, UK) for testing. Pilot experiments successfully demonstrated expression and basal activity of one of the *P. agathidicida* GPCR-PIPK's in yeast, suggesting this system is viable.

4.3 Discussion

4.3.1 The Chemotactic Behaviour of *P. agathidicida* to Amino Acids and Sugars

Phytophthora have been previously reported to be attracted to amino acids and simple sugars (Dukes and Apple, 1961; Khew and Zentmyer, 1973; Suo *et al.,* 2016). There are however differences in attraction towards amino acids depending on the species of *Phytophthora*. Acidic amino acids, most polar amino acids and some aromatic amino acids caused strong chemoattraction from *P. agathidicida* zoospores. In particular aspartate, glutamate, asparagine, glutamine and phenylalanine were the strongest attractants out of the amino acids tested. Aspartate, glutamate, asparagine and glutamine have all been reported to be attractants to various other *Phytophthora* species before (Khew & Zentmyer, 1973; Halsall, 1975; Morris & Ward, 1992; Suo *et al.,* 2016), however phenylalanine has not.

The aromatic amino acids produced an interesting result; phenylalanine and tryptophan caused strong chemoattraction but tyrosine did not. Tyrosine and phenylalanine only differ by a single hydroxyl group on phenyl ring on tyrosine. The presence of this hydroxyl group

makes the side chain slightly more polar in comparison with phenylalanine; this slight difference was enough to change the chemotaxis response of *P. agathidicida* zoospores. The response is most likely receptor mediated, where the receptor's ligand is a non-polar aromatic amino acid. All other non-polar amino acids did not produce a significant response, indicating there is no chemoreceptor for these amino acids present on *P. agathidicida* zoospores.

There were also some disparities between the polar amino acids. All polar amino acids except cysteine caused chemotaxis attraction by *P. agathidicida.* This indicates that the presence of the thiol group must be interfering with the chemotactic response of *P. agathidicida* zoospores.

The basic amino acids arginine and histidine have been shown to cause attraction in other *Phytophthora* species before (Khew and Zentmyer, 1973; Suo *et al.,* 2016) however *P. agathidicida* zoospores showed no significant attraction to basic amino acids.

Non-surprisingly *P. agathidicida* zoospores were strongly attracted to the simple sugars glucose, galactose, fructose, lactose and sucrose. Sucrose attracted nearly 3 times more zoospores then glucose and fructose, indicating that this disaccharide caused a stronger attraction then the simpler sugars. Plants continuously generate sucrose and it is present in high levels in the phloem vessels (Arnold, 1968) so is not surprising that it is a strong chemoattractant. Similar results have been seen in *S. cerevisiae* (Lamaire *et al.*, 2004) but *P. sojae* had similar chemotaxis levels across all the sugars (Suo *et al.,* 2016).

The positive control (10% cV8) is a mixture of amino acids, sugars and organic acids, however it was noticed that on average a range of 200-400 zoospores swam into the capillaries, which is significantly more zoospores than any single amino acid or and sugar tested. This suggests that synergism between compounds may be occurring which causes a higher level of attraction of the zoospores (Halsall, 1975). Testing compounds together could produce a stronger attraction from *P. agathidicida* zoospores, for example ethanol or sucrose in combination with amino acids have been shown to drastically increase chemotaxis in other species of *Phytophthora* (Halsall, 1975).

Compounds that eliut a chemotactic response do not necessarily promote germination or mycelial growth (Dukes and Apple, 1992). Arginine, aspartate and asparagine were tested to see if *P. agathidicida* germination rates differed between the amino acids. The germination rates for all three amino acids were less than the negative control (water), even though aspartate and asparagine are strong chemoattractants for *P. agathidicida*. Chemoattractants are not always related to uptake of the nutrient for *Phytophthora* (Erwin & Ribeiro, 1996).

4.3.2 *P. agathidicida* Zoospores are Attracted to Low pH Conditions

To test if changing the pH of amino acids affects the chemotaxis response of *P. agathidicida*, glutamate and arginine were re-assayed at pH 3.0 - 11.0. At pH 3.0 both amino acids strongly attracted *P. agathidicida* zoospores; this is in contrast to the assays conducted at pH 11.0, in which no chemoattraction was observed.

Kauri substantially influences the surrounding soil with its leaf litter, which generates an acidic soil environment. A mature kauri can form an acidic soil layer of pH 3.1 up to 2 meters in depth (Wyse and Burns, 2013). This pH would allow the amino acids to be in the more favoured ionic structure and thus act as strong chemoattractants for *P. agathidicida* zoospores. This acidic environment generated by kauri could be a key component of the attraction of *P. agathidicida* zoospores towards their host.

The enhanced chemotaxis behaviour of zoospores to amino acids in lower pH environments is consistent with results produced by other researchers on various *Phytophthora* species (Halsall, 1975; Khew and Zentmyer, 1973). Cationic amino acids have been shown to be strong attractants to other *Phytophthora* zoospores before which is thought to be influenced by the slight negative charge on the surface of the zoospores, causing them to be more attracted to positive or neutral environments and compounds (Khew and Zentmyer, 1973; Halsall, 1975).

It has been reported before that *Phytophthora* zoospores tend to migrate away from regions with a high hydrogen ion concentration (Halsall, 1975). Water controls with adjusted pH values were tested to see if just changing the hydrogen ion concentration affected chemotaxis of *P. agathidicida.* However the format of the chemotaxis assay only tests for

compounds which cause attraction, not repulsion. There was no attraction to any of the pH adjusted water controls but it is unknown if there was repulsion. If *P. agathidicida* is also repelled by hydrogen ions then the repellent nature of the hydrogen ions was counteracted by the attraction of the amino acids which was maximal at low pH. Further experiments should be conducted to test whether pH or certain amino acids caused the zoospores to repel. These additional experiments would clarify the relationship between the zoospores and the acidic environment and also could give some interesting results, giving us a chemotaxis repertoire of not only attractants but also repellents.

4.3.3 PIPK is Involved in *P. agathidicida* Chemotaxis

Chemotaxis is initiated through the activation of chemoreceptors. To better understand *P. agathidicida's* chemotaxis behaviour there needs to be a better understanding of their chemoreceptors and the subsequent pathways which initiate flagella movement. *Phytophthora* chemoreceptors are GPCR's, some of which have a novel PIPK associated with them. It is hypothesised that upon activation GPCR-PIPKs are able to trigger heterotrimeric G-protein signalling and phosphoinositide second-messenger synthesis which initiates movement of the zoospore flagella either towards or away from the chemical signal (Bakthavatsalam *et al.,* 2006; Hua *et al,* 2015). To test this theory *P. agathidicida* zoospores were exposed to PIPK inhibitors. Zoospores exposed to the PIPK inhibitor GSK2126458 were motile but no longer exhibited chemotaxis towards the positive control (Table 4.2). This confirmed the hypothesis that PIPK is required for zoospore chemotaxis and must play a key role in propagating the ligand signal within the cell.

4.3.4 Expression Trials of *P. agathidicida* PIPK

The chemotaxis assays from 4.2.4 showed that PIPK is integral in zoospore chemotaxis. Characterisation of this novel protein could help understand the mechanism of zoospore chemotaxis. Attempts were made to express and purify a tr-PIPK for future characterisation experiments. However, the expression trials conducted in this research were not successful as the PIPK protein was not very soluble. Proteins associated with GPCRs are notoriously difficult to express (Grisshammer, 2009). Attempts were made to increase the solubility of

PIPK by decreasing the temperature, changing media, using alternative strains and expressing with chaperones. However, codon bias, mRNA stability, protein folding, phosphorylation, and glycosylation are just some of the factors which can cause eukaryotic proteins to be expressed insolubly (Khow & Suntrarachun, 2012). In reality time constraints meant these factors could not be tested in detail. Trialling techniques which aid in eukaryotic post-translational modification could also be conducted in the future, as well as DNA analysis of the tr-PIPK insert for the presence of rare codons which could result in mistranslational amino acid substitutions, frameshifting or premature translational termination (Khow & Suntrarachun, 2012).

4.3.5 Identifying Ligands for *P. agathidicida* GPCRs

Knowing what *P. agathidicida* is attracted to gives some indication of what chemoreceptors may be present on the zoospores. From chemotaxis assays with amino acids and sugars (4.2.1) we can hypothesis that *P. agathidicida* zoospores have a receptor for sugars with a higher affinity for sucrose compared with the other sugars tested. We can also hypothesise that there are receptors specific for acidic amino acids, most polar amino acids and non-polar aromatic amino acids, with a strong affinity for amino acids at pH 3.0.

These hypotheses can be tested using molecular based analysis on *P. agathidicida's* GPCRs. A yeast-based reporter system can be used to identify ligands for GPCRs from a range of different species (Ladds *et al.*, 2003; Tehseen, 2014). This system is now going to be applied to the study of GPCRs from *P. agathidicida*. Two GPCRs were chosen to be used for this system, GK4 and GPR11, which have been identified to be important in chemotaxis and zoospore development in other *Phytophthora* species (Wong *et al.*, 2010; Yang *et al.*, 2013). The system is not currently available in New Zealand but Dr. Graham Ladds (University of Cambridge, UK) has agreed to test the *P. agathidicida* GK4 and GPR11 GPCRs. Therefore plasmids containing the GPCRs of interest were constructed (4.2.6). *P. agathidicida's* genomic DNA was extracted and the genes for GK4 and GPR11 receptors were isolated and inserted into the plasmids required for the yeast-based reporter system. These constructs will be used in future experiments to identify some of the key ligands which initiate chemotaxis and zoospore development in *P. agathidicida.*

Chapter 5

Finding Potential Inhibitors of *P. agathidicida* using a High Throughput Screen

The results described in this chapter represent the author's contributions to the following publication:

Lawrence, S., **Armstrong, C.**, Patrick, W., and Gerth M. (2017) High-Throughput Chemical Screening Identifies Compounds that Inhibit Different Stages of the *Phytophthora agathidicida* and *Phytophthora cinnamomi* Life Cycles. *Frontiers in Microbiology*. 8, 1340-1350

All work presented in this chapter is the author's own work, unless specifically stated otherwise. Work completed in conjunction with the first author of the publication (Scott Lawrence) is stated.

5.1 Introduction

5.1.1 Finding a Cure to Kauri Dieback Disease

Managing *Phytophthora* is notoriously difficult and controlling the disease can simply come down to three options: finding resistant host strains, adjusting the environmental conditions against the favour of *Phytophthora* or applying a chemical treatment (Erwin & Ribeiro, 1996). With kauri dieback, the foremost aim is to protect the ancient taonga trees already in our forest. Since relying on finding resistant kauri is futile for the fate of our ancient kauri and controlling the environment in a natural ecosystem is not suitable, chemical control will be the most appropriate solution for saving kauri. A chemical treatment that could be used to cure an already infected kauri has yet to be discovered.

5.1.2 Developing a high throughput screen for *P. agathidicida*

There are many reports of screening common fungicides *in vitro* against other species of *Phytophthora* (Akrofi *et al.*, 2013; Elliott *et al.*, 2015; Saville *et al.*, 2015) however there has been no report of using a high throughput method to screen large libraries of compounds that could contain a new treatment for *Phytophthora*. Common *Phytophthora* disinfectants such as TriGene®, Virkon®, Phytoclean™, Janola® and Citricidal® have been previously tested on *P. agathidicida* (Bellgard *et al.*, 2010) with various successes; however to our knowledge there has not been a large scale screen of any other disinfectants or compounds against *P. agathidicida*. With the disease spreading at such a fast rate, a high throughput screen was developed in this research with the aim of potentially discovering a cure for *P. agathidicida* and to further our understanding of this relatively new pathogen. The screen initially focused on compounds which inhibit mycelial growth and then any short listed compounds were tested for their effects on other life stages.

5.2 Results

5.2.1 High Throughput Screen

The screening process used a modified Kirby-Bauer disk diffusion assay (Bauer *et al.*, 1966) with potential anti-oomycete compounds located near the edge of the agar on filter paper discs and a mycelial agar plug placed in the centre of the plate (Figure 5.1). The library of potential anti- oomycete compounds used in this screen came from Biolog Phenotype Microarray (PM) plates (Bochner *et al.*, 2001); the full list of compounds is presented in Appendix I. These PM plates contain 120 compounds chosen to test the chemical sensitivity of fungi. Although *Phytophthora* are taxonomically different to fungi, they are fungus-like. Both *Phytophthora* and fungi grow as filamentous mycelia in their vegetative state, as well as both forming asexual and sexual spores, making them morphologically similar and therefore it was thought that these compounds were a good starting point for the screen (Latijnhouwers *et al.*, 2003).

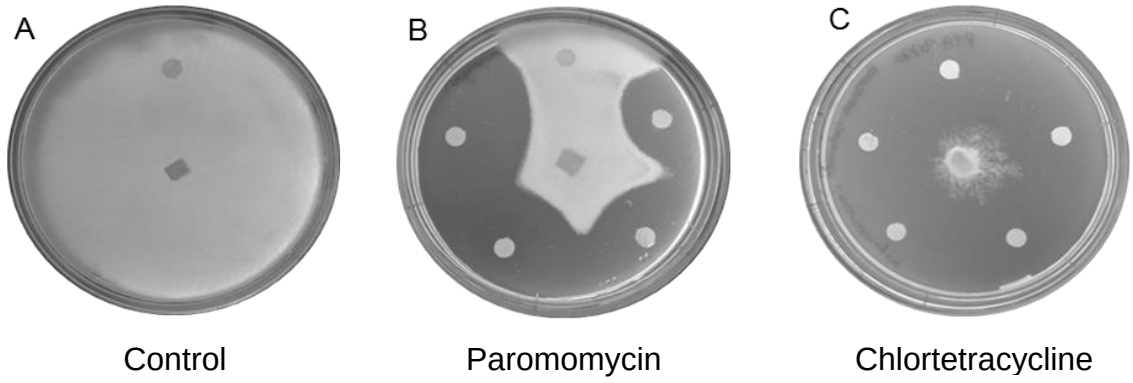

Figure 5.2: High throughput screen for anti-oomycte compounds. Potato Dextrose Agar (PDA) plates have a control filter paper disk (water) at the top of each control (A) and test plates (B & C). The test plates have four more filter paper disks of a potential anti-oomycte compound which increases in concentration going clockwise from the control filter paper at the top. PDA plates were inoculated with an agar plug of *P. agathidicida* and left to grow for 6 days at 22°C. (A) Control plate with a uniform circular lawn of mycelium. (B) Paromomycin causes zones of inhibition around the inhibitor containing filter papers. (C) Chlortetracycline hydrochloride completely inhibited mycelial growth

Out of the 120 compounds, 14 compounds caused some form of disruption to mycelial growth of *P. agathidicida* (Table 5.1). Copper salts, glycine hydrochloride, cobalt chloride, sodium and doxycycline hyclate had the least obvious growth inhibition but still slowed the

mycelium growth rate relative to the control. Paromomycin, kanamycin, tobramycin, fluorodeoxyuridine, pentamide isethionate, cadmium chloride and benzethonium chloride caused obvious zones of inhibition around the disks containing the compound (Figure 5.1B). Chlortetracycline and tetrazolium violet were the most effective compounds as they completely stopped mycelial growth (Figure 5.1C).

Table 5.2: Compounds discovered to disrupt mycelial growth of *P. agathidicida* 3770 from the high-throughput screen.

Compound	Inhibitor Type
Paromomycin	Aminoglycoside antibiotic
Kanamycin	Aminoglycoside antibiotic
Tobramycin	Aminoglycoside antibiotic
Fluorodeoxyuridine	Antimetabolite oncology drug
Pentamidine Isethionate	Antiprotozoal drug
Benzethonium Chloride	Antiseptic
Tetrazolium Violet	Reducer / oncology drug
Chlortetracycline	Tetracycline antibiotic
Doxycycline Hyclate	Tetracycline antibiotic
Sodium Arsenite	Anionic toxin
Cobalt (III) Chloride Hexahydrate	Cationic toxin
Glycine Hydrochloride	Amino Acid
Copper (II) Chloride	Cationic toxin
Copper (II) Sulfate	Cationic toxin

Five out of the fourteen compounds that had an inhibitory effect were antibiotics. Three of these antibiotics were aminoglycosides (kanamycin, paromomycin and tobramycin) and the other two were tetracycline antibiotics (chlortetracycline and doxycycline hyclate), both of which are protein synthesis inhibitors, which disrupt translation on the bacterial ribosomes.

There were sixteen anionic toxins in the PM screen and only arsenate showed an inhibitory effect on *P. agathidicida*. There were however three cationic toxins that showed inhibitory effects (cobalt and the copper salts).

In the PM screen there were seven common antifungals: triazoles (fluconazole, myclobutanil, propiconazole), an imidazole (miconazole), a polyene antimycotic (nystatin), a nucleoside analog (5-fluorocytosine) and cycloheximide, none of these showed any inhibitory effects. None of the chelating agents tested, such as dipyridyl, EDTA, and EGT, caused any inhibition either.

5.2.2 Quantitative Analysis of High Throughput Screen Compounds

Of the fourteen inhibitory compounds from the high-throughput screen, eight of the most promising inhibitors were selected for further quantitative testing. To evaluate how effective these compounds were, EC_{50} values were determined for the inhibition of mycelial growth on *P. agathidicida* strains 3813 and 3815, as well as the standard strain 3770 (Table 5.2). Concentration values were log-transformed and a normalised non-linear regression curve was generated which resulted in well fitted EC_{50} curves that have R^2 values > 0.85 (Figure 5.2).

Compounds which were not tested further include sodium arsenite and fluorodeoxyuridine (an oncology drug) which showed promising inhibitory effects; however due to their high toxicity levels and their unsustainability for use in the environment this meant they were excluded from further analysis. Doxycycline hyclate and pentamidine isethionate were not available in the laboratory so were not evaluated further. Lastly, tobramycin and glycine hydrochloride needed very high concentration ranges (>512 µg/mL) to get an EC_{50} value, therefore they were also excluded from further analysis (data not shown).

The most effective mycelial growth inhibitor across all three strains was chlortetracycline, with an average EC_{50} value of 0.75 µg/mL. The aminoglycosides: kanamycin and tobramycin were the least effective inhibitors, with EC_{50} values as high as 50 µg/mL from *P. agathidicida* 3815.

Table 5.3: EC_{50} values for inhibition of mycelial growth on *P. agathidicida* strains 3770, 3813 and 3815 against the high throughput screen compounds. The mean of three biologically replicated assays was used to determine the EC_{50} values presented. The numbers in the brackets are the 95% confidence interval from the three independent replicates.

Inhibitory Compounds from High Throughput Screen	EC_{50} values of *P. agathidicida* strains (µg/mL)		
	3770	**3813**	**3815**
Chlortetracycline Hydrochloride	0.88 (0.69 – 0.90)	0.88 (0.75 – 1.0)	0.59 (0.49 – 0.69)
Tetrazolium Violet	1.1 (3.6 – 5.9)	1.1 (0.86 – 1.4)	0.90 (0.71 – 0.92)
Paromomycin Sulfate	4.3 (4.0 – 4.6)	1.9 (1.8 – 2.1)	1.1 (0.85 – 1.3)
Benzethonium Chloride	3.2 (2.3 – 3.5)	3.0 (2.7 – 3.2)	1.8 (1.7 – 2.0)
Copper (II) Chloride	6.8 (6.5 – 7.2)	6.7 (5.2 – 8.5)	3.6 (2.9 – 4.5)
Copper (II) Sulfate	9.0 (8.4 – 9.6)	8.1 (6.7 – 9.7)	3.3 (2.1 – 4.8)
Cobalt Chloride	11 (7.0 – 18)	21 (16 – 27)	26 (23 – 31)
Kanamycin Sulfate	28 (22– 35)	16 (12 – 22)	50 (38 – 67)

The sensitivity of the three P. agathidicida strains were similar for the eight compounds. The EC_{50} values for each strain varied slightly however for most compounds this variation was not substantial (Table 5.2). The *P. agathidicida* strain 3815 had the lowest EC_{50} values for all inhibitors, except for the cobalt chloride and kanamycin.

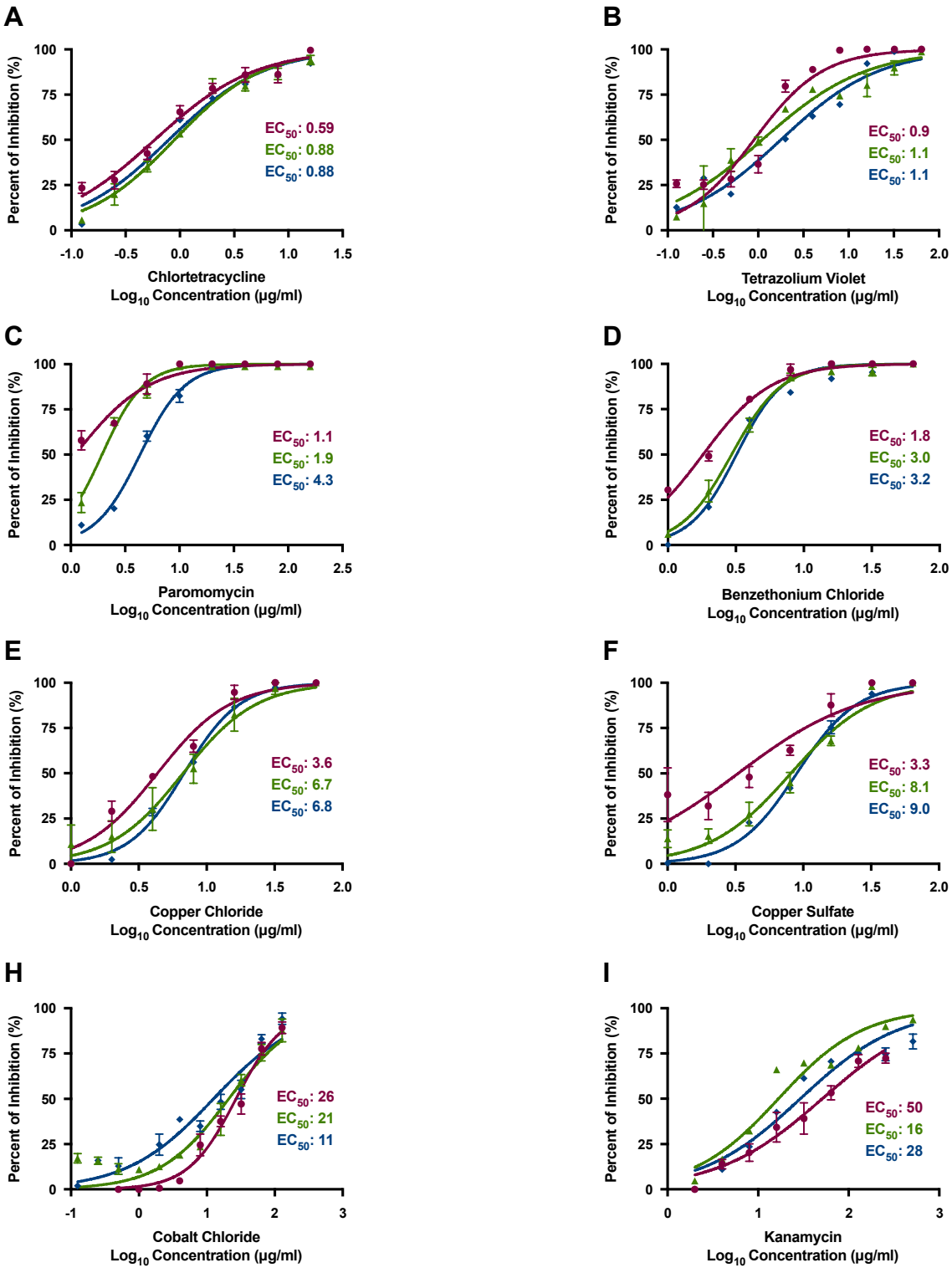

Figure 5.3: Mycelial growth inhibition curves for *P. agathidicida* strains 3770, 3813 and 3815. Each curve (A–I) shows the inhibition curves for a single inhibitory compound, as stated in the x-axis label. Each data point is a mean of the percentage of inhibition from three independent assays. The standard error of the mean is represented as bars from each data point. The EC_{50} values determined from these graphs are shown in each panel.

5.2.3 Quantitative Analysis of Current *Phytophthora* Treatments

One of the more effective inhibiting compounds from the screen was a quaternary ammonium, benzethonium chloride. Quaternary ammonium salts, in particular benzalkonium chloride, have been shown to inhibit mycelial growth of *Phytophthora* before (Noske and Shearer, 1985). Benzalkonium chloride is also found in TriGene®, a disinfectant used at foot washing stations to help stop the spread of kauri dieback. The EC_{50} values of benzalkonium chloride and TriGene® against *P. agathidicida* mycelial growth, germination and zoospore motility were analysed. As well as these products, other disinfectants used on *Phytophthora* or similar diseases were tested for their effectiveness at inhibiting *P. agathidicida.* These disinfectants included, Virkon®, Agrifos-600 and "Compound X".

5.2.3.1 Mycelial Growth

The calculated EC_{50} values for all disinfectants on mycelial growth are presented in Table 5.4. These values varied widely; benzalkonium chloride had a similar EC_{50} value (1.8 µg/mL) to benzethonium chloride (Table 5.3), whereas TriGene® and "Compound X" had EC_{50} values > 10^4. All inhibition curves were fitted with a regression analysis, with all curves having R^2 values > 0.9 (Appendix III).

Table 5.4: EC_{50} values for inhibition of mycelial growth by current *Phytophthora* treatments. The mean of three biologically replicated assays was used to determine the EC_{50} values presented. The numbers in the brackets are the 95% confidence interval from the three independent replicates.

Disinfectant	**EC_{50} values of *P. agathidicida* mycelial growth (95% confidence interval)**
Agrifos-600	3.0 (1.7 - 5.3) µg/mL
Benzalkonium chloride	1.8 (1.6 - 2.1) µg/mL
Virkon®	0.060 (0.027 - 0.12) %
TriGene®	1.0×10^{-4} (0.61×10^{-4} - 1.62×10^{-4}) %
Compound "X"	0.14×10^{-4} (0.12×10^{-4} - 0.15×10^{-4}) µg/mL

5.2.3.2 Zoospore Motility

To determine the effect of the compound on zoospore motility, the minimum concentration of the compound which stopped the zoospores from moving after 5 minutes of exposure was recorded (Table 5.4). Virkon® and TriGene® were the most effective compounds, stopping zoospore motility at a concentration of 0.005%. "Compound X", although effective at inhibiting mycelial growth (Figure 5.3), was not effective at inhibiting zoospore motility.

Table 5.5: Inhibition of zoospore motility by current *Phytophthora* treatments. Minimum concentrations of disinfectant required to cause zoospores to become immotile within 5 min of treatment. [1]the lowest concentration tested therefore minimum concentration required for motility inhibition may be lower.

Disinfectants	**Minimum Concentration for Zoospore Immobility**
Agrifos-600	0.5 µg/mL
Benzalkonium chloride	0.1 µg/mL
Virkon®	0.005 % [1]
Trigene®	0.005 % [1]
Compound "X"	100 µg/mL need to repeat

5.2.3.3 Zoospore Germination

To evaluate the compounds effect on *P. agathidicida* zoospore germination, EC_{50} values were calculated using the same analysis method as mycelial growth EC_{50} values (Table 5.5). The compound's germination EC_{50} values were similar to the mycelial growth EC_{50} values. TriGene® and "Compound X" were very effective at inhibiting germination rates with (EC_{50} values > 10^{-4}). All inhibition curves were fitted with a regression analysis, with all curves having R^2 values > 0.9 (Appendix IV).

Table 5.6: EC_{50} values for inhibition of zoospore germination by current *Phytophthora* treatments. The mean of three biologically replicated assays was used to determine the EC_{50} values presented. The numbers in the brackets are the 95% confidence interval from the three independent replicates.

Disinfectants	**EC_{50} values of *P. agathidicida* germination (95% confidence interval)**
Agrifos-600	1.6 (0.66 – 3.9) µg/mL
Benzalkonium chloride	0.5 (0.43 – 0.60) µg/mL
Virkon®	0.009 (0.007 - 0.012) %
Trigene®	0.7×10^{-4} (0.36×10^{-4} - 1.1×10^{-4}) %
Compound "X"	13×10^{-4} (10×10^{-4} - 17×10^{-4}) µg/mL

5.3 Discussion

5.3.1 High Throughput Screen

This is the first time a high-throughput screen of potential *Phytophthora* inhibitors has been reported for *P. agathidicida*. The need for a treatment for this disease is becoming paramount for the survival of kauri, a screen like this, which can now be used to test many more libraries of potential anti-oomycete compounds, will be very useful in finding a cure.

The fungal chemical sensitivity libraries from Biolog were useful for initial screening, although they did have some limitations. The Biolog PM plates have different concentration ranges for each compound and the concentration values are not specified. Without knowing the concentrations of the compounds, the compounds cannot be compared with each other for their effectiveness of inhibition. For example, glycine hydrochloride and tobramycin were false positives; from the screen they were detected to cause inhibition; although these compounds did cause inhibition the concentration values needed to generate EC_{50} values were too high for them to ever be applicable in the field. Although it is not known if there were any false negatives, it would seem unlikely as the concentration ranges given in the PM plates seemed to be wide enough that it would include a strong enough concentration to show inhibitory effects on *P. agathidicida*, just like with glycine hydrochloride and tobramycin. Although the compounds in the plates PM21D-25D were used to test the sensitivity of fungal cells, not *Phytophthora*, the PM plates did include a lot of common antibiotics and antimicrobial compounds, none of which have been reported before on *P. agathidicida,* so it was still a good initial library to screen.

5.3.2 Anti-oomycete Compounds: Mycelial Growth

5.3.2.1 Inhibitors from the Screen

Chlortetracycline, tetrazolium violet, benzethonium chloride and paromomycin were the most effective compounds at inhibiting mycelial growth of *P. agathidicida*, with chlortetracycline being the most effective with an average EC_{50} value of 0.75 µg/mL. Glycine hydrochloride and tobramycin were the least effective inhibitors; their growth

inhibition curves did not fit in the eight fold dilution concentration range and their R^2 was <0.5 (data not shown), for this reason they were not studied further. Kanamycin and cobalt chloride were also ineffective compounds, with the high EC_{50} values of 31 and 19 µg/mL respectively.

Five of the eight potential inhibitors identified from the screen were antibiotics, with three being aminoglycosides. Although the mode of action of aminoglycosides on prokaryotes is well known, it is not well understood how they affect eukaryotes, however there are many reports of aminoglycosides inhibiting *Phytophthora* and other closely related eukaryotes (Palmer & Wilhelm, 1978; Edlind, 1989; Lee *et al.*, 2005). Chlortetracycline, a tetracycline antibiotic, was the most successful at inhibiting *P. agathidicida,* with an average EC_{50} value of 0.75 µg/mL, and has been previously shown to be just as effective in inhibiting *P. cinnamomi* mycelial growth (Leary *et al.*, 1982). Paromomycin was also very effective at inhibiting *P. agathidicida,* with an average EC_{50} value of 2.4 µg/mL. A similar result with paromomycin has also been seen in other *Phytophthora* species both *in vitro* and *in vivo* (Lee *et al.*, 2005). Paromomycin has successfully been used in trials on chilli pepper plants against *P. capsici* which have been cured of the disease without any negative effect on the plant (Balaraju *et al.*, 2016). Antibiotics have been used to control bacterial plant pathogens on commercial crops since the 1950's, but the emergence of antibiotic resistance has restrained the use of certain antibiotics in agriculture (McManus *et al.*, 2002). Antibiotic resistant *Phytophthora* have already been grown *in vitro* (Ann and Ko, 1992) and with the possibility of asexual transfer of genetic material between strains of *Phytophthora*, the use of antibiotics in the environment is debatable (Shattock and Shaw, 1976).

As far as we know, this is the first report of tetrazolium violet inhibiting *Phytophthora*. Tetrazolium was successful at inhibiting *P. agathidicida* across the life cycle, causing zoospore motility to stop at 0.25 µg/mL, as well as efficiently inhibiting zoospore germination (EC_{50} = 3.2 µg/mL) and mycelial growth (EC_{50} = 0.9-4.7 µg/mL). Tetrazolium violet is predominantly used in microbiological studies as a redox indicator for cellular respiration. High concentrations of tetrazolium violet have been reported to inhibit bacteria when using it as a redox reagent (Junillon *et al,* 2014) and it has recently been tested for its potential as an oncology drug. Although its direct mechanism of inhibition is not yet

known, it has been reported to induce apoptosis in tumour cells by increasing the activity of caspases (Chai *et al.,* 2009; Zhang *et al.,* 2012). Interestingly, from the screen, tetrazolium violet did not cause inhibition against *P. cinnamomi* (Lawrence, 2017) indicating that *P. agathidicida* is for some reason more susceptible to this compound. All other compounds from the screen were within an order of magnitude between the two species. Further EC_{50} tests need to be completed on *P. cinnamomi* before a conclusion about differences in the species can be stated. Further research into tetrazolium violet needs to be done to know how it is inhibiting *P. agathidicida* and whether or not *P. agathidicida* is more susceptible to this compound.

Of the eight compounds that showed inhibition of mycelial growth, none have been reported to be inhibitors for *P. agathidicida* before. Copper salts and quaternary ammonium salts have been reported previously as treatments of other *Phytophthora* diseases. Copper based fungicides have been a traditional treatment of *Phytophthora* since 1885 (Millardet, 1885; Leach, 1966; Howard *et al.,* 1997; Govers *et al.,* 2017). Copper treatments are currently being used in New Zealand's forestry industry to treat Dothistroma needle blight which is destroying pine plantations (Crockett, 1976; Bulman *et al.,* 2013), and to provide protection against *Pseudomonas syringae pv. Actinidiae*, a deadly kiwi-fruit pathogen (Gould, 2015). Copper treatment, although successful at controlling certain plant pathogens, is known to accumulate in soils, reducing soil fertility, and is also known to have phytotoxic effects in some plants (Wightwick *et al.,* 2008; Kiaune & Singhasemanon, 2011).

Benzethonium chloride, a quaternary ammonium salt, had a low EC_{50} value of 2.7 µg/mL on average across the three strains. Another quaternary ammonium salt, benzalkonium chloride, is used in a commercial product called Phytoclean. Phytoclean is used as a management solution for *Phytophthora* species in Australia, such as a disinfectant to reduce the spread of *P. cinnamomi* (Hall *et al.,* 2003). Benzalkonium chloride had a lower EC_{50} value of 1.8 µg/mL (Table 5.3) showing that it is slightly more efficient at killing *P. agathidicida* mycelium than benzethonium chloride. This quaternary ammonium salt can be found in basic household cleaners as well as being one of the active ingredients in TriGene®, a disinfectant against bacteria, fungi and viruses. TriGene® was one of the most

successful disinfectants tested, with an EC_{50} value of 1.0 x 10^{-4} %. TriGene® (2%) is currently being used as a prevention method for further spread of the kauri dieback disease, where the solution is placed at wash stations at the start of a track or forestry area containing kauri trees. TriGene® is known to kill three of the four life stages of *P. agathidicida* (mycelia, sporangia and zoospores) and can successfully kill *P. agathidicida* in soil, thus reducing the rate of infection (Bellgard *et al.*, 2010). Knowing that this high throughput screen was able to pick out similar compounds already being used on *P. agathidicida* and compounds that are being used on other *Phytophthora* species, gives us confidence to use this as a valid method to find new cures for the disease.

5.3.2.2 Currently used Disinfectants

Having established a protocol to calculate EC_{50} values of compounds for mycelial growth inhibition, the next rational step was to see how currently-used disinfectants or recommended disinfectants would affect *P. agathidicida.* As said above, benzalkonium chloride and TriGene® were very effective inhibitors, similar results for *P. agathidicida* have been reported before (Bellgard *et al.*, 2010) so the results were not surprising but it was important to validate. Virkon® is used in the laboratory at 1% concentration to wash and sterilise glass ware which has been in contact with *P. agathidicida* and therefore it was not surprising for it to also have a low EC_{50} value of just 0.06%. Phosphite, an anionic form of phosphorous acid (HPO_3^{2-}) is a common treatment for *Phytophthora* diseases worldwide (Coffey & Bower, 1984; Hardy *et al*., 2001). Phosphite is also being trialled as a potential short-term solution for kauri dieback disease (Horner and Hough, 2013). Agrifos-600, a commercial product containing phosphite, had an EC_{50} value of 3.0 µg/mL (Table 5.3) as well as an effective germination EC_{50} value (1.6 µg/mL). Compared with the other disinfectants however these values are quite high, phosphite is known to boost plants' immune responses, so it is to be expected that *in vitro* studies of phosphite on *Phytophthora* will be understating its effects *in planta*. "Compound X" was also very efficient at inhibiting *P. agathidicida* except for zoospore motility, however, this result was only repeated once and further replicates are required before drawing any conclusions. "Compound X" has been successfully used in agriculture for mildew and this result could

mean it could be looked into further as a potential chemical treatment for kauri dieback disease.

5.3.3 Anti-oomycete Compounds: Targeting the Whole Life Cycle

Following on from these results, Scott Lawrence and I then tested these eight "winning" compounds on the other stages of the *P. agathidicida* life cycle. Zoospore motility and zoospore germination against the compounds was assessed (Lawrence *et al.*, 2017). The effectiveness of the compounds varied greatly between the different stages. The antibiotics, although very effective at inhibiting mycelial growth, were very poor at inhibiting zoospore motility and germination. Chlortetracycline for example was the most effective against mycelial growth however it was not effective at stopping zoospore motility and had no effect on zoospore germination. Kanamycin also had no effect on zoospore motility and germination. Paromomycin was the only antibiotic to prevent zoospore motility, however the concentration was still high (150 µg/mL). Antibiotics have previously been reported to inhibit *P. infestans* mycelial growth, and like *P. agathidicida*, the inhibition by the antibiotics was not as successful on other life cycle stages (Érsek, 1975). The copper salts and benzalkonium chloride were the most effective across all three assays and life stages, indicating their promising use to help manage the disease.

5.3.4 Strain Variability

Phytophthora strains, belonging to the same species, have been reported previously to vary in sensitivity to different chemicals and fungicides (Elliott *et al.*, 2015; Saville *et al.*, 2015); this is the first time strain variation has been studied in *P. agathidicida*. The EC_{50} values for the *P. agathidicida* strains were overall similar, with the exception of *P. agathidicida* 3815 which were somewhat more sensitive to the inhibitory compounds, especially towards the copper salts (Table 5.2). Overall, the sensitivity of the strains towards the inhibitory compounds were alike. It has however been reported that strains from the same species of *Phytophthora* vary markedly in their sensitivity to phosphite. Different *Phytophthora* strains can have a high or a low affinity uptake to phosphite which affects their sensitivity to the fungicide (Coffey & Joseph 1985; Wilkinson *et al*., 2001). It would therefore be wise to still test *P. agathidicida* strain sensitivity against any future inhibitory compounds,

especially if an active ingredient is phosphite. Differences in antibiotic sensitivity between *Phytophthora* species has been reported before; however, it is also thought the differences between strains is due the differences in conditions the strains have been exposed too, such as nutrient levels (Leary *et al.*, 1982). The three strains used in this experiment were subjected to the same culturing conditions as each other, so any differences observed here are not due to differences in the culturing. It is also important to note that each strain grew at different rates to each other, so when calculating the EC_{50} values, each strain was compared with their own strain control. Emerging resistances to antibiotics and phosphite has also been reported both *in vitro* and *in planta* and it is therefore important to keep monitoring strain variability for resistance if a treatment is to be used in the future for kauri dieback (Shattock, 2002). This test was important to conduct for future management of the disease, as the results suggest that the same treatment could potentially be used in kauri trees infected with different strains.

Chapter 6

Discussion

6.1 Significance of this Work

6.1.1 Zoospore Production and Chemotaxis Behaviour of *P. agathidicida*

As an emerging pathogen, much remains to be discovered about *P. agathidicida*. Compared with more established *Phytophthora* species such as *P. cinnamomi* (Chen & Zentmyer, 1970), at the onset of this project there were few protocols available for studying *P. agathidicida* in a laboratory setting.

One of the first challenges was to develop a reliable method for zoospore production. The conditions for zoospore production have been optimised in other, more well-studied *Phytophthora* species. However, the exact conditions for zoospore production are species specific and had never been optimised for *P. agathidicida*.

In this research a reliable method for the increased production of sporangia and zoospores for *P. agathidicida* was developed (Chapter 3). The number of sporangia produced was greatly improved by using autoclaved soil extracts to wash and incubate mycelium in. Light exposure was also found to be vital for stimulating asexual spore production. Furthermore, passaging through a fleshy fruit like pear was the best way to preserve the pathogenicity of *P. agathidicida* when routinely transferring between cV8 plates. Combined, these changes to the protocol improved zoospore production enough to begin exploring the chemotactic behaviour of *P. agathidicida* zoospores.

In Chapter 4, the chemotactic behaviour of *P. agathidicida* zoospores was explored. Zoospores of other *Phytophthora* species are known to be attracted to common plant exudates such as amino acids and sugars (Suo *et al.,* 2016). However, there is variation amongst *Phytophthora* species. Overall twenty four chemicals (*e.g*: amino acids, sugars) were screened for potential chemo-attraction. Sucrose, acidic amino acids, most polar amino acids and most aromatic amino acids caused strong chemoattraction from *P. agathidicida* zoospores. This attraction is enhanced when amino acids are in low pH environments, at a pH level similar to the soil conditions around kauri (Wyse & Burns, 2013).

6.1.2 Newly Developed Anti-oomycete High Throughput Screen

Despite all that has been discovered about *Phytophthora*, approaches on curing the disease is still at work. Currently the primary disease management strategy for agricultural crops is to breed resistance (Erwin & Ribeiro, 1996); however this is not a viable strategy to protect plants already in the environment, like kauri. It is also futile to rely on resistant kauri strains in nature as the genetic diversity is already low and fragmented after extensive logging in the 19th century (Steward & Beveridge, 2010). Kauri will become extinct unless a permanent treatment is discovered. The existing management is focused on public awareness and hygiene procedures to help reduce the spread and impact of dieback (Bellgard *et* al., 2009). Currently there are trials using phosphorous acid on infected kauri, however this will merely slow down the rate of infection and will not cure it or prevent it spreading (Horner, *et al.,* 2013). Thus a more effective treatment is required to control *P. agathidicida* for the protection of the kauri tree. A chemical treatment is paramount to cure and protect the kauri that already stand.

In this research a high throughput screen for anti-oomycete compounds for their effectiveness against mycelial growth was developed. This was the first time a high throughput screen has been developed for anti-oomycete compounds. Overall, a 120 compounds were screened using a high throughput assay for mycelial growth inhibition on *P. agathidicida.* Leads from the high throughput screening were then followed-up in zoospore motility and germination assays. In total, eight inhibitory compounds for *P. agathidicida* were identified; most of which had been reported as an anti-oomycete compound before. The EC_{50} values for mycelial growth inhibition were all within an order of magnitude between the different *P. agathidicida* strains tested, suggesting that they have a similar level of sensitivity to the compounds and therefore similar treatment could be applied regardless of the strain. Some compounds had different efficiencies at inhibiting the different life stages of *P. agathidicida*; antibiotics for example were good at inhibiting mycelial growth however were very poor at inhibiting zoospore motility and germination. This shows that all stages of *P. agathidicida's* life cycle have to be considered for future treatment development. The development of the screen opens up opportunity for many

more potential compounds to be screened so treatment for kauri dieback and other *Phytophthora* diseases can be discovered rapidly.

6.2 Future Directions

6.2.1 Exploring the Chemosensory Repertoire of *P. agathidicida*

Chemotaxis assays can be used to screen compounds for a chemotaxis response from zoospores. Issues with producing sufficient zoospore numbers at the beginning of this research (Chapter 3) meant only preliminary chemotaxis experiments were conducted for this research. The compounds which were initially studied at were nonspecific plant compounds such as amino acids and sugars. Many more compounds such as vitamins, flavones, phenolic compounds, nitrogen sources, nucleotides, growth regulators, organic acids, other common plant compounds could also be tested in the future for their chemotactic response. Understanding the entire chemosensory repertoire of *P. agathidicida* could be extremely useful for the treatment and prevention of the disease in kauri. For example, if a key compound(s) is found to cause strong attraction for *P. agathidicida* then a potential solution could be to establish decoy plants that release the compound(s) into the soil to reduce the soil population of *P. agathidicida* around kauri (Morris & Ward, 1992).

As well as identifying what attracts *P. agathidicida* zoospores, what repels them is of equal interest. Chemotaxis assays can also be set up to test for repellents for future experiments. Any compounds found to repulse zoospores could be used in the field as a potential repellent around kauri which could stop the spread of the disease.

Root exudates are the metabolites that are released from the roots of a plant into the surrounding soil (Badri & Vivanco 2009). Root exudates from host plants have been shown to initiate chemotactic responses of zoospores in most host specific soil-borne *Phytophthora* species, and are thought to be critical for the pathogen to locate their hosts and initiate attack (Zentmyer, 1961; Khew & Zentmyer, 1973). Chemical analysis of root exudates alongside chemotaxis assays could identify the compounds exuded from kauri roots which cause attraction of *P. agathidicida*. Untargeted metabolic profiling of root

exudates using ultra-high-pressure liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF mass spectrometry) has been used to identify a wide range of metabolites from other plants (Melnik *et al.*, 2017; Petriacq *et al.*, 2017). This technique could potentially be used on root exudates, and the identified compounds could be validated in chemotaxis assays. Research using kauri root exudates could answer why *P. agathidicida* zoospores infect kauri and why some kauri are more susceptible to infection than others. This approach could also be extended to profile companion plants of kauri. More research into the chemotaxis response of non-host exudates could reveal potential inhibitory or repellent plants, which could be planted near non infected kauri to create a natural barrier for the disease.

This thesis mainly focused on the chemotactic response of zoospores, but of equal interest are the encystment and germination patterns of zoospores. For example, chemotropism influences the direction and orientation of the germ tube which helps the *Phytophthora* find an optimal infection site (Judelson & Blanco, 2005). Previous studies have shown zoospore chemotaxis, encystment and germination can be stimulated by different external compounds (Suo *et al.*, 2016). The germination rates of *P. agathidicida* zoospores were briefly looked into in this research (4.2.3) which showed amino acids which caused chemo-attraction did not improve germination rates, indicating that external compounds can initiate and affect different zoospore behaviour. Similar results were seen when exposing zoospores to anti-oomycete compounds; some compounds affected zoospore motility and germination differently (5.2.3 and 5.3.3). Future experiments should also explore zoospore encystment rates, germination rates and chemotropism of the germ tube to potential chemoattractants and chemorepellents.

6.2.2 Identifying Ligands for *P. agathidicida* GPCRs

Phytophthora are eukaryotic microbes, and as such, use GPCRs for chemosensing. Two GPCRs have been identified to be important in chemotaxis and zoospore development in other *Phytophthora* species before: GK4 and GPR11 (Wong *et al.*, 2010; Yang *et al.*, 2013). A yeast-based reporter system is capable of identifying ligands for exogenous GPCRs. This system uses genetically engineered *Schizosaccharomyces pombe* strains that contain Gα-transplants that enables exogenous GPCRs to be expressed. When an

exogenous GPCR is activated by a ligand this activates the pheromone response pathway and leads to the expression of a signal-dependent *lacZ* reporter gene (Ladds *et al.*, 2003).

The yeast-based reporter system has been used to identify ligands for GPCRs from a range of species, including the worm *C. elegans* (Tehseen, 2014) and humans (Ladds *et al.*, 2003), but it has never been applied to the study of *Phytophthora*. The system is not currently available in New Zealand but Dr. Graham Ladds (University of Cambridge, UK) has agreed to test the *P. agathidicida* GK4 and GPR11 GPCRs.

As part of this research, four reporter plasmids: p425GPD-GK4, p425GPD-GPR11, p426GPD-GK4 and p426GPD-GPR11, were successfully constructed. Pilot experiments conducted by Dr. Graham Ladds (University of Cambridge, UK) used these reporters and successfully demonstrated expression and basal activity of one of the *P. agathidicida* GPCR-PIPK's in yeast. This suggests this system could be viable and can be used to identify the ligands which activate *P. agathidicida'*s GPCRs.

6.2.3 Anti-oomycete Compounds for Oospores

In Chapter 5, a screen was developed to look for inhibitory compounds against mycelial growth in *P. agathidicida*. Any inhibitory compounds found from this screen were then assessed on multiple stages of its life cycle. *Phytophthora* have a well-adapted life cycle with different spores, all of which vary in their form and possibly their response to an inhibitory compound. When developing a compound to kill *Phytophthora,* all stages of the life cycle need to be assessed. However, the effects of the inhibitory compounds on oospores were not tested in this thesis. Oospores have thick cell walls and are known to live for long periods of time and are able to survive during extreme conditions, making them a resilient part of *Phytophthora's* life cycle (Erwin & Ribeiro, 1996). Common disinfectants such as Trigene (2% w/v) or Phytoclean (10% w/v), cannot kill *P. agathidicida* oospores, despite the rest of the life stages of *P. agathidicida* being successfully inhibited by these disinfectants (5.2.3; Bellgard *et al,* 2010). Finding a cure for kauri dieback disease therefore needs to include analysis of anti-oomycete compounds on oospores, as well as the rest of *Phytophthora's* life cycle.

6.3 Conclusion

Overall it is clear more research needs to be done to save kauri. However the studies described in this thesis represent key advancements in our ability to culture the organism in the laboratory, and provides a new insight into its chemotactic behaviour and ways to control it.

The high throughput screening method developed herein is a platform technology for rapidly identifying potential anti-oomycete compounds in the future. This research also highlighted the need to evaluate anti-oomycete across multiple life stages of *Phytophthora.* However, a great deal remains to be understood about the molecular mechanisms that underpin how *P. agathidicida* infects kauri. Such knowledge is essential if novel compounds are to be developed to target this organism.

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Appendices

Appendix I: Biolog Phenotype Microarray Plates 21D-25D

Compounds in the Biolog Phenotype Microarray plates used in this study

Type	Name	Biolog PM	Wells
Anion	Potassium chromate	Plate PM24C	D5-D8
Anion	Sodium (*meta*)perioda	Plate PM23A	E5-E8
Anion	Sodium arsenite	Plate PM23A	E9-E12
Anion	Sodium benzoate	Plate PM24C	B9-B12
Anion	Sodium cyanate	Plate PM23A	F9-F12
Anion	Sodium cyanide	Plate PM23A	H5-H8
Anion	Sodium dichromate	Plate PM21D	D9-D12
Anion	Sodium fluoride	Plate PM24C	F9-F12
Anion	Sodium metaborate te	Plate PM23A	E1-E4
Anion	Sodium metasilicate	Plate PM24C	C5-C8
Anion	Sodium metavanadate	Plate PM22D	A5-A8
Anion	Sodium nitrite	Plate PM23A	G1-G4
Anion	Sodium orthovanadat	Plate PM23A	G5-G8
Anion	Sodium selenate	Plate PM23A	H1-H4
Anion	Sodium selenite	Plate PM21D	G1-G4
Anion	Sodium thiosulfate	Plate PM23A	H9-H12
Antibioti	4-nitroquinoline-*N*-ox	Plate PM25D	C9-C12
Antibioti	Alexidine	Plate PM25D	D1-D4
Antibioti	Aminacrine	Plate PM24C	A5-A8
Antibioti	Apramycin sulfate	Plate PM24C	A1-A4
Antibioti	Blasticidin hydrochlo	Plate PM24C	B1-B4
Antibioti	Bleomycin	Plate PM22D	F9-F12
Antibioti	Bleomycin	Plate PM22D	H1-H4
Antibioti	Chloroalanine hydroc	Plate PM25D	B1-B4
Antibioti	Clomiphene citrate	Plate PM25D	E9-E12
Antibioti	D-cycloserine	Plate PM21D	F9-F12
Antibioti	D,L-serine hydroxama	Plate PM22D	C9-C12
Antibioti	Dequalinium chloride	Plate PM23A	B5-B8
Antibiotic	Fluconazole	Plate PM24C	G9-G12
Antibiotic	Hygromycin B	Plate PM25D	D5-D8
Antibiotic	Ibuprofen	Plate PM25D	G9-G12
Antibiotic	Kanamycin monosulfate	Plate PM25D	B9-B12
Antibiotic	Miltefosine	Plate PM22D	C5-C8
Antibiotic	Neomycin	Plate PM21D	F5-F8

Antibiotic	Paromomycin	Plate PM22D	H5-H8
Antibiotic	Pentamidine isethionate	Plate PM24C	C9-C12
Antibiotic	Polymyxin B	Plate PM22D	D1-D4
Antibiotic	Tamoxifen	Plate PM24C	H5-H8
Antibiotic	Thialysine	Plate PM24C	D9-D12
Antibiotic	Thioridazine hydrochloride	Plate PM24C	B5-B8
Antibiotic	Tobramycin	Plate PM25D	A5-A8
Antibiotic	Triclosan	Plate PM22D	B9-B12
Antibiotic	Trifluoperazine	Plate PM21D	G9-G12
Antibiotic	Zaragozic acid A	Plate PM24C	A9-A12
Cation	Aluminum sulfate	Plate PM24C	G5-G8
Cation	Ammonium sulfate	Plate PM23A	A9-A12
Cation	Cadmium chloride hydrate	Plate PM23A	B1-B4
Cation	Chromium (III) chloride hexahydrate	Plate PM23A	D1-D4
Cation	Cobalt (II) chloride hexahydrate	Plate PM23A	D5-D8
Cation	Copper (II) sulfate	Plate PM21D	F1-F4
Cation	Cupric chloride dihydrate	Plate PM23A	D9-D12
Cation	Dodecyltrimethyl ammonium bromide	Plate PM21D	B5-B8
Cation	Lithium chloride	Plate PM22D	F5-F8
Cation	Magnesium chloride	Plate PM21D	E9-E12
Cation	Manganese (II) chloride	Plate PM21D	E5-E8
Cation	Nickel chloride	Plate PM21D	G5-G8
Cation	Palladium(II) chloride	Plate PM25D	G5-G8
Cation	Thallium(I) acetate	Plate PM22D	G9-G12
Cation	Zinc chloride	Plate PM21D	H9-H12
Chelator	1-hydroxypyridine-2-thione	Plate PM21D	D1-D4
Chelator	2,2′-Dipyridyl	Plate PM21D	A5-A8
Chelator	BAPTA	Plate PM22D	E5-E8

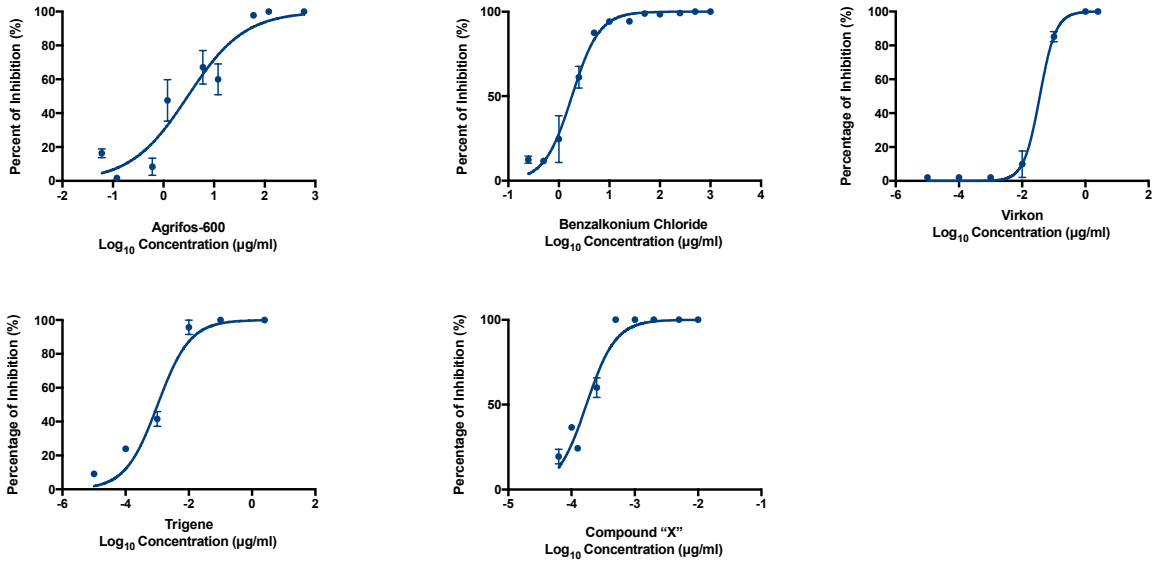
Appendix II: Chemical Analysis of Ross Creek Pond Water

Chemical analysis of the pond water used for *P. agathidicida* sporangium production. Analysis was carried out by Citilab, Dunedin, New Zealand

Chemical	Concentration (g/m^3)
Bicarbonate	65
Calcium (dissolved)	9.54
Magnesium (dissolved)	4.56
Nitrate	0.24
Nitrate-N	0.05
Nitrite	<0.1
Nitrite-N	<0.05
Phosphate	<0.1
Phosphate-P	<0.05
Potassium (dissolved)	1.78
Sulfate	1

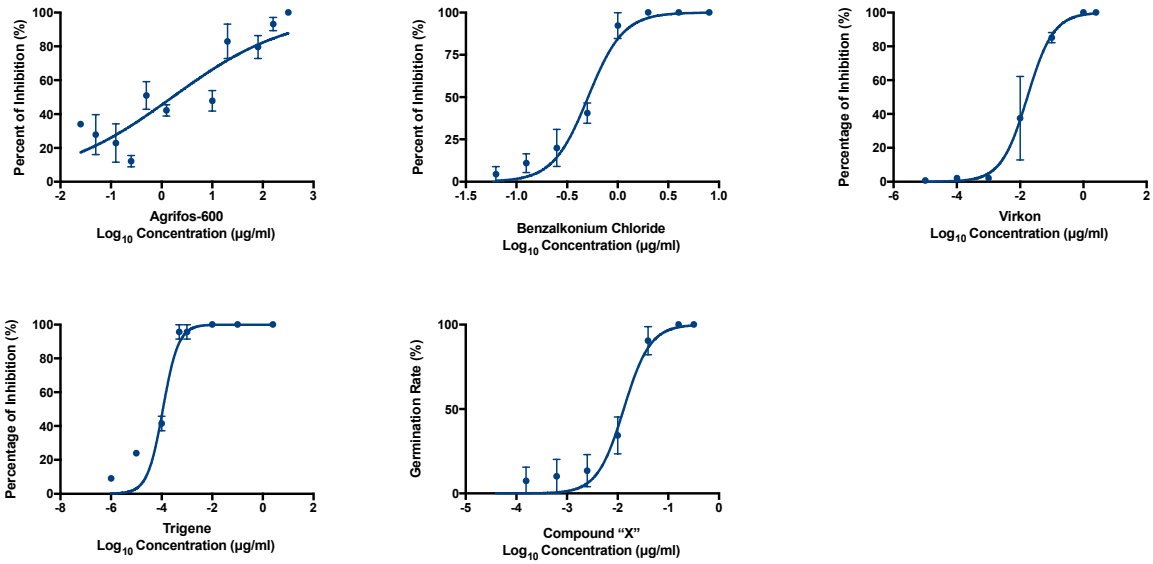
Appendix III: EC_{50} Curves of Mycelial Growth against Disinfectants

Mycelial growth inhibition curves for *P. agathidicida* 3770 against current and potential treatments



Appendix IV: EC_{50} Curves of Germination Rates against Disinfectants

Germination inhibition curves for *P. agathidicida* 3770 against current and potential treatments



Appendix V: Publication

Lawrence, S., **Armstrong, C**., Patrick, W., and Gerth M. (2017) High-Throughput Chemical Screening Identifies Compounds that Inhibit Different Stages of the *Phytophthora agathidicida* and *Phytophthora cinnamomi* Life Cycles. *Frontiers in Microbiology*. 8, 1340-1350

frontiers in Microbiology

ORIGINAL RESEARCH
published: 19 July 2017
doi: 10.3389/fmicb.2017.01340

High-Throughput Chemical Screening Identifies Compounds that Inhibit Different Stages of the *Phytophthora agathidicida* and *Phytophthora cinnamomi* Life Cycles

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OPEN ACCESS

Edited by:
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Reviewed by:
Santa Olga Cacciola, University of Catania, Italy
Giles E. St. J. Hardy, Murdoch University, Australia

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Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Microbiology

Received: *15 March 2017*
Accepted: *03 July 2017*
Published: *19 July 2017*

Citation:
Lawrence SA, Armstrong CB, Patrick WM and Gerth ML (2017) High-Throughput Chemical Screening Identifies Compounds that Inhibit Different Stages of the Phytophthora agathidicida and Phytophthora cinnamomi Life Cycles. Front. Microbiol. 8:1340. doi: 10.3389/fmicb.2017.01340

Oomycetes in the genus *Phytophthora* are among the most damaging plant pathogens worldwide. Two important species are *Phytophthora cinnamomi*, which causes root rot in thousands of native and agricultural plants, and *Phytophthora agathidicida*, which causes kauri dieback disease in New Zealand. As is the case for other *Phytophthora* species, management options for these two pathogens are limited. Here, we have screened over 100 compounds for their anti-oomycete activity, as a potential first step toward identifying new control strategies. Our screening identified eight compounds that showed activity against both *Phytophthora* species. These included five antibiotics, two copper compounds and a quaternary ammonium cation. These compounds were tested for their inhibitory action against three stages of the *Phytophthora* life cycle: mycelial growth, zoospore germination, and zoospore motility. The inhibitory effects of the compounds were broadly similar between the two *Phytophthora* species, but their effectiveness varied widely among life cycle stages. Mycelial growth was most successfully inhibited by the antibiotics chlortetracycline and paromomycin, and the quaternary ammonium salt benzethonium chloride. Copper chloride and copper sulfate were most effective at inhibiting zoospore germination and motility, whereas the five antibiotics showed relatively poor zoospore inhibition. Benzethonium chloride was identified as a promising antimicrobial, as it is effective across all three life cycle stages. While further testing is required to determine their efficacy and potential phytotoxicity *in planta*, we have provided new data on those agents that are, and those that are not, effective against *P. agathidicida* and *P. cinnamomi*. Additionally, we present here the first published protocol for producing zoospores from *P. agathidicida*, which will aid in the further study of this emerging pathogen.

Keywords: oomycetes, *Agathis australis*, kauri dieback, avocado root rot, phenotype microarray, zoospore, high-throughput screening

INTRODUCTION

Species in the genus *Phytophthora* (from the Greek for "plant-destroyers") are among the most serious threats to native plants and horticultural species alike, with a global economic impact estimated in the billions of dollars per annum (Erwin and Ribeiro, 1996). While they superficially resemble filamentous fungi, *Phytophthora* are actually oomycetes and therefore more closely related to diatoms and brown algae in the stramenopiles (Gunderson et al., 1987; Thines, 2014). *Phytophthora* live in plant tissues, soil, or water, and under favorable conditions of temperature, moisture, and nutrition produce sporangia, followed by the release of motile zoospores, allowing infection to spread rapidly among host plants (Judelson and Blanco, 2005).

Two species of particular importance in New Zealand are *Phytophthora agathidicida* and *Phytophthora cinnamomi*. *P. agathidicida* (formerly *Phytophthora* taxon agathis or PTA) is a root and collar/stem canker pathogen responsible for dieback in kauri (*Agathis australis*), an iconic tree that is native to New Zealand (Beever et al., 2009; Weir et al., 2015). Kauri is a keystone species that has a profound influence on the surrounding soil, canopy, and biodiversity (Ecroyd, 1982; Wyse et al., 2014). Kauri dieback was first recognized over 40 years ago (Gadgil, 1974) and is continuing to spread through the forests of northern New Zealand, rapidly killing trees of all ages and sizes (Beever et al., 2009). Currently, there are no established treatment or control options for *P. agathidicida*, although the use of phosphite as a potential treatment for infected trees is being explored (Horner and Hough, 2013; Horner et al., 2015).

Phytophthora cinnamomi is a generalist pathogen capable of infecting thousands of different host plants in wildlands, horticulture and commercial nurseries, resulting in root rot and often death of the host plant (Erwin and Ribeiro, 1996). In New Zealand, the most economically important host is avocado (*Persea americana*). Globally, its principal food crop hosts are avocado and pineapple, but it is also an important pathogen of ecologically significant native plants throughout the temperate regions of the world. Some of the greatest plant losses are in areas with a Mediterranean climate including parts of the United States, Australia, Mexico, and the Iberian Peninsula (Burgess et al., 2017). It also infects a range of ornamental trees and shrubs and is readily spread through the movement and out-planting of nursery stock (Erwin and Ribeiro, 1996). Disease caused by *P. cinnamomi* has significant economic impacts on forestry and horticulture; in California alone, the annual loss from *P. cinnamomi* infections in avocado groves is US$30 million (Erwin and Ribeiro, 1996). *P. cinnamomi* has been called the "biological bulldozer" for its capacity to destroy plants (Carter, 2004), and it was recently identified as one of the top 10 oomycete pathogens, based on its scientific and economic importance (Kamoun et al., 2015).

Exacerbating the effects of *Phytophthora* infection is the fact that these species are notoriously difficult to control. While *Phytophthora* share many traits with the true fungi, they are phylogenetically distinct enough to lack many of the canonical molecular targets of labeled fungicides (Rolando et al., 2016). For example, *Phytophthora* species lack the biosynthetic pathways for ergosterol and a chitin-based cell wall. The development of resistance is also an ongoing problem (Cohen and Coffey, 1986; Parra and Ristaino, 2001; Gisi and Sierotzki, 2008; Miao et al., 2016). Once an area becomes infected, eradication can require chemical treatment, physical barriers to prevent disease spread, and destruction of host plants (Dunstan et al., 2010). Environmental toxicity of chemical treatments and the logistics of application further complicate disease management and eradication efforts.

In this study, we have sought to identify anti-oomycete compounds that inhibit mycelial growth, zoospore germination, and/or zoospore motility in *P. agathidicida* and *P. cinnamomi*, as a first step toward development of more effective control agents. By using phenotype microarray (PM) plates (Bochner et al., 2001) as sources of potential antimicrobials, we screened 120 known antimicrobial chemicals in a high-throughput fashion. Here, we explore the chemical susceptibility of the emerging pathogen, *P. agathidicida*, and compare its susceptibility profile to these chemicals with the more well-characterized species, *P. cinnamomi.*

MATERIALS AND METHODS

Routine Culturing

Phytophthora agathidicida isolate NZFS 3770 and *P. cinnamomi* isolate NZFS 3910 were sourced from Scion (Rotorua, New Zealand). The two species were routinely cultured at 22°C in darkness on clarified 20% V8-juice agar (V8A). For antibiotic susceptibility testing using PM plates (Biolog Inc, CA, United States), isolates were cultured on potato-dextrose agar (PDA; *P. agathidicida*) or cornmeal agar (CA; *P. cinnamomi*) (Becton, Dickinson & Co, NJ, United States). EC_{50} assays were carried out using CA for both species.

Anti-oomycete Compound Screening

The activities of potential anti-oomycete compounds were tested using a modified version of the Kirby–Bauer disk diffusion assay (Bauer et al., 1966). Test compounds were prepared by adding 20 μL of sterile water to each well of a PM plate. Each plate (PM 21D, 22D, 23A, 24C, and 25D) contains 24 different compounds at four different concentrations. Five microliters of each resuspended compound were added to sterile 6 mm filter disks (Whatman number 1 paper). After sufficient drying, the four filter disks containing a single test compound at different concentrations were placed at regular intervals around the edges of a PDA or CA plate, along with a control disk (sterile water). Each plate was then inoculated by placing a 3 mm agar plug of mycelium (taken from the leading edge of an actively growing mycelial mat) in the center. Each plate was incubated at 20°C in the dark and radial growth was measured after 5 days (*P. cinnamomi*) or 7 days (*P. agathidicida*). Three biological replicates (from independently grown cultures) were performed for each compound screened. Any compound that reproducibly gave zones of inhibition that were greater than the control disk and were dose-dependent was scored as a positive.

Determination of EC_{50} Values for Inhibition of Mycelial Growth

The EC_{50} values for radial growth inhibition were determined for each compound that inhibited *Phytophthora* growth in the disk diffusion assays. CA was amended with eight twofold dilutions of the compound of interest, with concentrations ranging from 0.125 to 512 μg/ml. Concentration ranges were chosen based on preliminary growth inhibition assays and differed among compounds. Control plates (CA only) were included for each compound tested, and three biological replicates were performed for each compound and concentration. Each plate was inoculated by placing a 3 mm agar plug of mycelium (taken from the edge of an actively growing mycelial mat) in its center; plates were then incubated at 20°C for 4 days (*P. cinnamomi*) or 6 days (*P. agathidicida*). Two perpendicular measurements were taken for each plate and averaged, and the size of the agar plug was subtracted to obtain the final measurement of radial growth. Measurements from test compound plates were subtracted from control plate measurements and converted to inhibition percentages. To estimate EC_{50} values, compound concentrations were log-transformed, and non-linear regression with curve fitting (by least squares) was carried out using GraphPad Prism version 6.0. All chemicals were purchased from Sigma-Aldrich (MO, United States) except for copper sulfate pentahydrate (Ajax Chemicals, Sydney, Australia) and kanamycin sulfate (Applichem GmbH, Darmstadt, Germany).

Zoospore Germination and Motility

In order to produce sporangia in *P. cinnamomi* and *P. agathidicida*, approximately 2 cm^2 of agar from the edge of an actively growing mycelial mat was excised and transferred to a Petri dish containing a 1:10 dilution of carrot broth (*P. agathidicida*) or clarified 20% V8 broth (*P. cinnamomi*), cut into ∼1 mm^2 pieces and then incubated at 25°C in darkness for 24 h. The following day, the broth was removed and each dish was washed four times in sterile Chen–Zentmyer salt solution (for *P. cinnamomi*; Chen and Zentmyer, 1970) or sterile pond water (for *P. agathidicida*) for 45 min at a time on the bench (*P. agathidicida*) or on a rocking table (*P. cinnamomi*), followed by overnight incubation (without rocking) at 22°C under light. Following sporangium production, zoospore release was induced by removing the liquid and washing each dish three times with sterile Milli-Q water that had been cooled to 4°C. Each wash was for 20 min, with the first two at room temperature and the final wash at 4°C. Wash volumes were 15 ml per dish for the first two washes, and 10 ml for the final wash. Following the final wash, dishes were returned to room temperature for 30–90 min until sufficient numbers (a final concentration of approximately 1×10^3 zoospores/ml) of zoospores had been released.

To assess the effect of each putative anti-oomycete compound on zoospore germination, 200 μl of zoospore suspension were spread on triplicate water agar plates amended with the test compounds. Concentrations of the compounds were as for the mycelial growth inhibition assays, and ranged from 0.125 to 512 μg/ml. Plates were incubated for 12–16 h, and 100 zoospores per plate were counted under a Nikon C-DS dissecting microscope at 40× magnification to determine the percentage that had germinated. Spores were defined as having germinated if the germ tube length was at least twice as long as the spore diameter, as described previously (Liu et al., 2014). The level of germination inhibition was calculated by subtracting the percent germinated at each concentration from that of three control (water agar only) plates.

Zoospore motility assays were based on a method described previously (Hu et al., 2007). One milliliter of zoospore suspension (approximately 1,000 zoospores) was added to triplicate wells in a 24-well plate containing 10 μl of the test compound solution at different concentrations, resulting in five twofold dilutions of the test compound. Control wells were amended with an equal volume of sterile water. Zoospores were kept at room temperature, and observed with the dissecting microscope to determine the time required for all zoospore motility to stop. Observations were made at 5 min intervals for the first hour, then at 30 min intervals until motility had ceased. Wells that had no motile zoospores by the time of the first observation (5 min post-treatment) were scored as 0 (i.e., complete inhibition). Subsequent loss of motility was calculated by dividing the length of time zoospores remained motile by the average motility time in the controls.

RESULTS

High-Throughput Screening for Anti-oomycete Compounds

The activities of potential anti-oomycete compounds were tested using a disk diffusion assay (Bauer et al., 1966), where the zone of inhibition of mycelial growth around filter disks containing the test compounds was assessed (**Figure 1**). Each species was screened against a panel of Biolog PM plates. In total, the panel of five PM plates contained 120 potential antimicrobial compounds, with each compound present at four concentrations. The panel contained common anti-yeast and antifungal antibiotics, as well as other types of chemicals with antimicrobial activity such as metals, detergents, and inhibitors. The complete list of compounds tested is available in Supplementary Table 1.

Of the 16 anionic toxins tested, only one (arsenite) had an inhibitory effect. Similarly, only two of the 15 cationic toxins tested, copper (II) chloride and copper (II) sulfate, showed inhibitory effects. None of the chelating agents tested (including dipyridyl, EDTA, and EGTA) showed any inhibitory effects.

Several common antibiotics successfully inhibited growth, particularly at higher concentrations. The majority were aminoglycosides (hygromycin B, kanamycin, neomycin, paromomycin, and tobramycin), which act by inhibiting protein synthesis. Chlortetracycline (a tetracycline antibiotic which also inhibits protein synthesis) and D-cycloserine (an amino acid derivative which inhibits pyridoxal 5′-phosphate dependent enzymes) also inhibited mycelial growth. Of the antibiotics identified here, only neomycin and paromomycin have been previously reported to have anti-oomycete activity against other *Phytophthora* species (Lee et al., 2005).

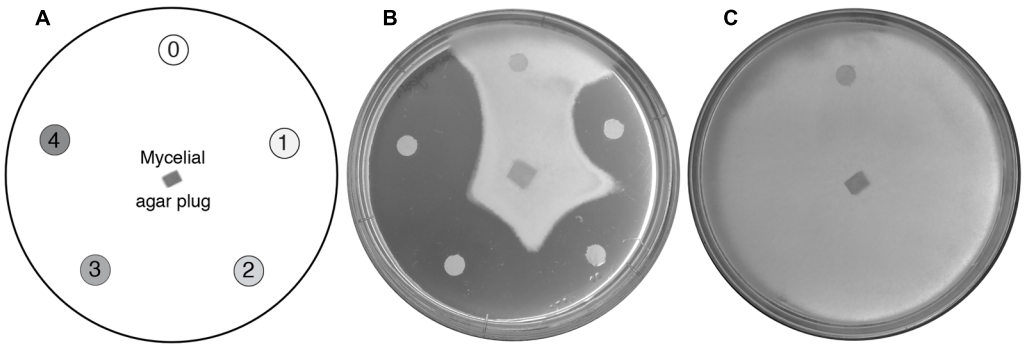

FIGURE 1 | High-throughput screening for anti-oomycete compounds. **(A)** Diagram showing the standard test arrangement. An agar plug of mycelium is used to inoculate an agar plate with disks saturated either with water (control; marked 0) or increasing concentrations of a test compound (in a clockwise direction, marked 1–4). **(B)** Representative results from a typical experiment. Here, *P. agathidicida* mycelial growth is inhibited in the presence of increasing concentrations of paromomycin. **(C)** Control plate showing normal growth of *P. agathidicida* in the absence of test compounds.

TABLE 1 | EC_{50} values for inhibition of mycelial growth.

	P. agathidicida **EC_{50} (μg/ml)**	***P. cinnamomi*** **EC_{50} (μg/ml)**
Benzethonium chloride	3.2 (2.3 – 3.5)	7.3 (6.5 – 8.3)
Chlortetracycline hydrochloride	0.79 (0.69 – 0.90)	1.1 (1.0 – 1.2)
Copper (II) chloride	6.8 (6.5 – 7.2)	3.2 (2.9 – 3.6)
Copper (II) sulfate	9.0 (8.4 – 9.6)	5.0 (4.5 – 5.5)
D-cycloserine	190 (150 – 240)	43 (37 – 49)
Kanamycin sulfate	28 (22 – 35)	52 (48 – 57)
Neomycin trisulfate	180 (170 – 190)	160 (140 – 170)
Paromomycin sulfate	4.3 (4.0 – 4.6)	8.5 (8.2 – 8.9)

Numbers in parentheses are 95% confidence intervals, determined from three independent experiments.

In addition to antibiotics, the PM screen included seven common antifungals: three triazoles (fluconazole, myclobutanil, propiconazole), an imidazole (miconazole), a polyene antimycotic (nystatin), a nucleoside analog (5-fluorocytosine) and cycloheximide. None of these showed any inhibitory effects.

Benzethonium chloride, a quaternary ammonium salt often used as a disinfectant, showed strong inhibition of *P. agathidicida* mycelial growth, and a low level of inhibition against *P. cinnamomi*.

Thallium (I) acetate and sodium arsenite both showed promising inhibitory effects; however, their non-selective toxicity (Hughes, 2002; Lennartson, 2015) makes them unsuitable for use in the environment so they were not tested further. Similarly, fluorodeoxyuridine (an oncology drug) and trifluoperazine (an antipsychotic) inhibited mycelial growth, but were excluded from further analysis due to their unsuitability for use in the real-world treatment of oomycete infections.

Mycelial Growth Inhibition

Based on the results of our qualitative high-throughput screen, we selected the eight most promising inhibitors for more quantitative testing. To begin, we determined EC_{50} values for inhibition of mycelial growth. This is important, because mycelial growth assays approximate both systemic growth within the plant, and across root-grafts between plants (i.e., root-to-root contact). Regression analysis resulted in well-supported inhibition curves, with all R^2 values >0.9 (**Figure 2**). The calculated EC_{50} values for all compounds are presented in **Table 1**. These values varied widely among the compounds tested, from 0.79 to 180 μg/ml.

The most effective inhibitor of mycelial growth in both *Phytophthora* species was chlortetracycline hydrochloride, with EC_{50} values of 0.79 and 1.1 μg/ml in *P. agathidicida* and *P. cinnamomi*, respectively. Benzethonium chloride was also an effective inhibitor, though more so for *P. agathidicida*, as was seen in the initial high-throughput screen. Both of the copper compounds tested had relatively low EC_{50} values, and were more effective against *P. cinnamomi*. EC_{50} values for the aminoglycoside antibiotics varied widely, with paromomycin by far the most effective. D-cycloserine was a relatively ineffective inhibitor, although it showed the greatest species specificity of any compound tested (almost fivefold more effective against *P. cinnamomi* than *P. agathidicida*).

Zoospore Germination Inhibition

In addition to mycelial growth, a useful antimicrobial agent could also inhibit one or more of the other stages in the *Phytophthora* life cycle (see, for example, Mircetich, 1970 and Judelson and Blanco, 2005). The first step in initiating a new infection is the encystment and germination of a zoospore on the host plant (Judelson and Blanco, 2005). We tested the ability of our eight candidate antimicrobials to inhibit zoospore germination, following a previous protocol (Liu et al., 2014).

As no previous studies have reported laboratory methods for producing *P. agathidicida* zoospores, several variations on the standard *P. cinnamomi* protocol were trialed in order to maximize the number of sporangia formed and zoospores released. It was found that excised pieces of mycelial mats grew best when grown in diluted, clarified carrot broth

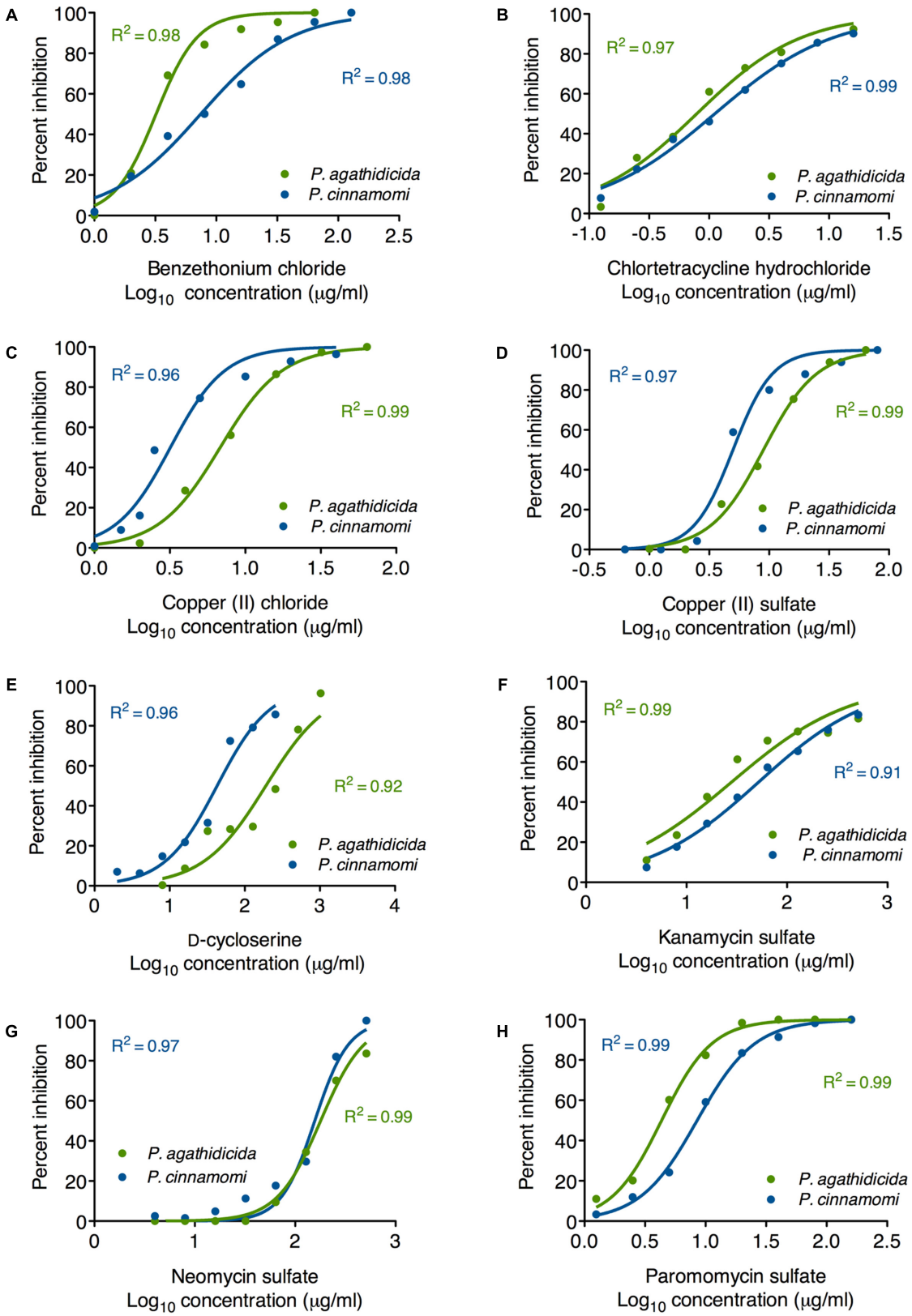

FIGURE 2 | Mycelial growth inhibition curves for *P. agathidicida* (green) and *P. cinnamomi* (blue). Each panel **(A–H)** shows the inhibition curves for a single compound, as identified in the label on the *x*-axis. Data points show the mean of triplicate inhibition assays.

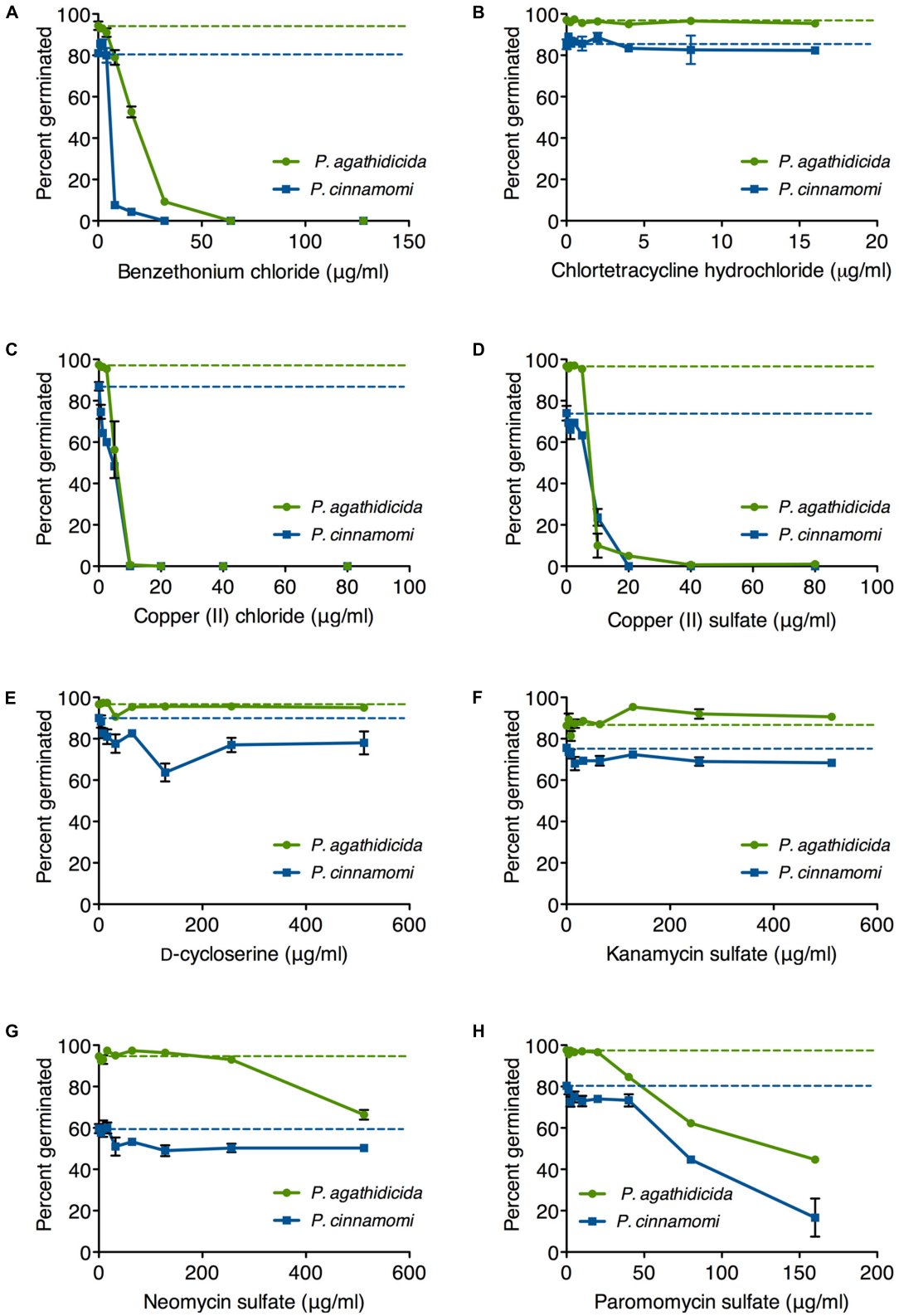

FIGURE 3 | Inhibition of zoospore germination in *P. agathidicida* (green) and *P. cinnamomi* (blue). Each panel **(A–H)** shows the inhibition curves for a single compound, as identified in the label on the *x*-axis. Dashed lines represent the germination rates on unamended agar. Data points are mean values from triplicate germination assays. Error bars are ± standard error of the mean. Where error bars are not visible, they are smaller than the symbol.

(Erwin and Ribeiro, 1996) rather than V8 broth. Sporangia production was increased when the subsequent washing steps were carried out using sterile (filtered and autoclaved) pond water (pH ~8.5) rather than the salt solution used for *P. cinnamomi*. The chemical composition of the pond water used can be found in Supplementary Table 2. Pond water samples from two sources (Rotorua, North Island, New Zealand and Dunedin, South Island, New Zealand) were trialed, with no noticeable differences in sporangium numbers. Adjusting the pH of the pond water up or down, to values between 5 and 10, reduced sporangia production, emphasizing that unadjusted pond water was optimal. As the mycelial mats of *P. agathidicida* were less adherent than those of *P. cinnamomi*, carrying out the wash steps on a rocking table tended to dislodge the mats and resulted in decreased numbers of sporangia; therefore wash steps were carried out without rocking. Methods for inducing zoospore release were as for *P. cinnamomi*, though *P. agathidicida* often required a longer final incubation step at room temperature before zoospores began to be released.

Germination rates on unamended agar plates ranged from 85 to 98% for *P. agathidicida* and 57–91% for *P. cinnamomi* (dashed lines in **Figure 3**). Germination of *P. agathidicida* zoospores was inhibited to a greater or lesser extent by five of the eight compounds tested (**Figure 3**), with the strongest inhibitors being benzethonium chloride (**Figure 3A**), copper (II) chloride (**Figure 3C**), and copper (II) sulfate (**Figure 3D**). All of these compounds inhibited germination completely at concentrations ranging from 10 to 80 μg/ml, with the two copper salts proving the most effective. On the other hand, neomycin and paromomycin only effected partial inhibition of germination at the highest concentrations tested (**Figures 3G,H**). The other antibiotics (chlortetracycline, D-cycloserine and kanamycin) did not inhibit germination of *P. agathidicida* zoospores.

The pattern was broadly similar for inhibiting germination of *P. cinnamomi* zoospores (**Figure 3**). As with *P. agathidicida*, the most effective inhibitors were benzethonium chloride (**Figure 3A**), copper chloride (**Figure 3C**), and copper sulfate (**Figure 3D**), all of which prevented germination completely at concentrations between 10 and 80 μg/ml. Paromomycin was the most effective of the antibiotics, although it only reduced the germination rate by fourfold (**Figure 3H**).

Zoospore Motility Inhibition

A third way of disrupting *Phytophthora* pathogenicity is to inhibit zoospore motility. Once released from sporangia, zoospores exhibit chemotaxis toward the roots of a new host plant, and, in the case of *P. cinnamomi*, can swim at approximately 0.5 m/h through water (Allen and Newhook, 1973; Allen and Harvey, 1974). Rendering them immotile would offer a novel containment approach that prevents spread from infected trees.

In control experiments, the untreated zoospores of *P. agathidicida* and *P. cinnamomi* remained motile in sterile water for approximately 17 and 20 h on average, respectively. We tested our set of eight anti-oomycete compounds over a range of concentrations. In general, we observed dose dependent responses, with higher compound concentrations leading to faster zoospore encystment. The only exception was D-cycloserine, which had no effect on the motility of *P. cinnamomi* zoospores at any concentration tested.

In order to quantify the effectiveness of the compounds, we compared the lowest concentration of each that was required to cause complete loss of motility within 5 min of treatment. The concentration required for this rapid inhibition varied widely among compounds (**Table 2**). Copper chloride, copper sulfate, and benzethonium chloride proved the most effective, with concentrations ≤0.1 μg/ml causing almost immediate loss of motility. At the other extreme, 256 μg/ml D-cycloserine was required to effect such immediate inhibition of *P. agathidicida* zoospore motility. Similarly, at the highest concentration tested (8 μg/ml), kanamycin failed to inhibit motility within 5 min. Instead, it took 25 min for *P. agathidicida* zoospores to lose motility at this concentration of kanamycin, and 60 min for *P. cinnamomi* zoospores. The general trends observed for both *Phytophthora* species in response to the eight compounds were similar, although *P. agathidicida* zoospores were over an order of magnitude more sensitive to neomycin than *P. cinnamomi* (**Table 2**).

DISCUSSION

This study provides the first comprehensive screening data on dozens of potential anti-oomycete compounds, for their effectiveness against two species of *Phytophthora*. In the case of *P. cinnamomi*, we have extended comparable early work on screening 13 fungicides (Smith, 1979) to incorporate a significantly broader range of compounds. For the emerging pathogen *P. agathidicida*, we have reported the first such high-throughput screen. By assessing effectiveness against three different stages of the *Phytophthora* life cycle, we have also explored new opportunities for control of these destructive organisms.

Of the 120 compounds screened, eight showed anti-oomycete activity against both *Phytophthora* species. *P. agathidicida* is an emerging pathogen, and none of the inhibitory compounds found here have been reported previously. The EC_{50} values for mycelial growth inhibition were all within an order of magnitude between the two species, suggesting that similar control options

TABLE 2 | Minimum concentrations of anti-oomycete compounds (in μg/ml) required to cause zoospores to become immotile within 5 min of treatment.

	P. agathidicida	*P. cinnamomi*
Benzethonium chloride	0.1	0.1
Chlortetracycline hydrochloride	16	8
Copper (II) chloride	0.025	0.05
Copper (II) sulfate	0.025	0.05
D-cycloserine	256	–
Kanamycin sulfate	–	–
Neomycin trisulfate	0.25*	4
Paromomycin sulfate	0.8	1.6

**This was the lowest concentration tested, therefore the minimum concentration required for immediate motility loss may be lower.*

may be available for *P. agathidicida* as those that are used for *P. cinnamomi*. However, further study of the various known isolates of *P. agathidicida* and *P. cinnamomi* will be necessary in order assess any potential differences in sensitivities.

Five of the eight anti-oomycete compounds identified in our screens were antibiotics that are more commonly associated with antibacterial activity. Antibiotics have been used since the 1950s to control bacterial diseases of various plants. However, with the growing threat of antibiotic resistance, this practice is the subject of debate (McManus et al., 2002; Tripathi and Cytryn, 2017; Wiles, 2017). The inhibitory abilities of the antibiotics identified in this study differed markedly between *Phytophthora* life cycle stages. This was particularly true of chlortetracycline, which was the most effective mycelial growth inhibitor in both species, but had no effect on zoospore germination rates and showed relatively poor inhibition of zoospore motility. Paromomycin was the only antibiotic tested here that inhibited all three life cycle stages, albeit with less efficacy in zoospores than the copper-containing compounds or benzethonium chloride. Overall, given the limited efficacy of these antibiotics against the different life cycle stages of *Phytophthora* and the potential for resistance to spread within and between species, our data suggest there is little potential for exploring these antibiotics as treatments for *Phytophthora* diseases, except perhaps as a last resort.

The most effective compounds across all three life cycle stages that we identified were copper salts and the quaternary ammonium salt benzethonium chloride. The identification of copper in our screens is unsurprising, as copper-based fungicides have traditionally been a mainstay of *Phytophthora* control (Keast et al., 1985; Howard et al., 1998; Gisi and Sierotzki, 2008). However, it is interesting to note that both copper chloride and copper sulfate were ∼2-fold less effective at inhibiting mycelial growth in *P. agathidicida* as compared to *P. cinnamomi*. In New Zealand, copper sprays have been used widely in agricultural, horticultural, and forestry settings to control a range of bacterial and fungal pathogens. For example, copper spraying has become a significant component of the kiwifruit industry's spray program for providing protection against infection by *Pseudomonas syringae* pv. *actinidiae* (Cameron and Sarojini, 2014; Gould et al., 2015). Similarly, copper fungicides are widely used by the New Zealand forestry industry for control of dothistroma needle blight (Bulman et al., 2013) and they have recently been shown to be effective against *Phytophthora pluvialis* (Rolando et al., 2016). However, concerns around soil accumulation and toxicity limit the use of copper sprays in natural environments (Wightwick et al., 2008; Komarek et al., 2010; Kiaune and Singhasemanon, 2011).

The quaternary ammonium salt benzethonium chloride was also effective across all three life cycle stages, for both species we examined. It showed EC_{50} values for mycelial growth inhibition of <10 μg/ml, immediate loss of zoospore motility at 0.1 μg/ml, and complete inhibition of germination at 32 μg/ml (*P. cinnamomi*) or 64 μg/ml (*P. agathidicida*). To our knowledge, this is the first report of the effectiveness of benzethonium chloride against any *Phytophthora* species, although other quaternary ammonium compounds have been shown to be effective against *P. cinnamomi* (Noske and Shearer, 1985). For example, benzalkonium chloride is sold in Australia under the brand name Phytoclean and is designed for use in shoe wash stations and for equipment/tool washdown. While preliminary, our data suggest that benzethonium chloride may be similarly useful, for example, in the shoe wash stations and sanitation kits that are currently employed to prevent the spread of kauri dieback. Before implementation, future work will need to include studies on phytotoxicity and the potential to be used as a soil drench, as was done during the commercialization of Phytoclean.

While our work expands the range of compounds known to possess anti-oomycete activity toward *P. cinnamomi*, it is particularly interesting with respect to *P. agathidicida*. As this species is an emerging pathogen that was only formally described in 2015 (Weir et al., 2015), comparatively little research has been carried out on treatment options. To date, phosphorous acid (phosphite) is the only chemical that has been used on kauri trees affected by *P. agathidicida*, and while this has proved an effective treatment, some signs of phytotoxicity were present at higher treatment concentrations (Horner and Hough, 2013; Horner et al., 2015). The EC_{50} for mycelial growth inhibition by phosphite was 4 μg/ml (Horner and Hough, 2013), similar to the EC_{50} we measured for benzethonium chloride (3.2 μg/ml). It must be noted, however, that phosphite acts both by inhibiting pathogen growth and by inducing host defense responses, therefore *in vitro* assays may overestimate the dose required *in planta* (Erwin and Ribeiro, 1996). Furthermore, copper chloride, copper sulfate, and benzethonium chloride all inhibited *P. agathidicida* zoospore motility and germination at low concentrations. Future work will also explore their effects on other stages of the *P. agathidicida* life cycle, such as the formation of the chlamydospores, oospores, and hyphal aggregates that are produced inside the roots of the hosts, as these may be critical targets for localized disease eradication.

Clearly work remains to be done to combat the threat to kauri posed by *P. agathidicida*. In this study, we have provided a list of promising anti-oomycete compounds, as well as a protocol for laboratory production of *P. agathidicida* zoospores. Our results suggest avenues for further investigation, bearing in mind that numerous factors must be considered before employing chemical control to manage plant disease, including: the rate, method, and frequency of application; possible phytotoxicity; and the potential for development of resistance. Ultimately, we hope that this study will facilitate further research into this pathogen, thereby helping to prevent the devastation of some of New Zealand's most iconic forests.

AUTHOR CONTRIBUTIONS

MG conceived this study. All authors participated in the experimental design, protocol development, and culturing of *Phytophthora*. SL and CA conducted all of the inhibition assays. All authors contributed to the writing and all authors have approved the final version.

FUNDING

This research was supported by a University of Otago Priming Partnerships grant and funding from the New Zealand National Science Challenge: New Zealand's Biological Heritage Ngā Koiora Tuku Iho.

ACKNOWLEDGMENTS

Phytophthora agathidicida isolate NZFS 3770 and *P. cinnamomi* isolate NZFS 3910 were provided by the Healthy Trees, Healthy Future Research Programme (Scion, Rotorua, New Zealand). We also gratefully acknowledge Drs Rebecca Ganley, Nari Williams, and Peter Scott for protocol advice and their assistance in establishing our *Phytophthora* research programme at the University of Otago.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01340/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.